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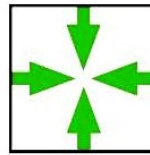
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**FONDAZIONE IRCCS
ISTITUTO NAZIONALE
DEI TUMORI**

Sistema Sanitario  **Regione
Lombardia**

Gut microbiota and trastuzumab response in HER2-positive breast cancer

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ABSTRACT

The use of trastuzumab as standard treatment for HER2-positive breast carcinomas (BCs) had largely improved the clinical outcome of BCs patients. Unfortunately, many of them do not respond to the therapy and the pathological complete response (pCR) is achieved in only 50% of patients even in those with tumours classified as the most sensitive to trastuzumab (i.e. HER2-enriched and high immune infiltration).

Given the relevance of the immune system in trastuzumab cytotoxicity and the immune regulatory functions of gut bacteria, this project aims at investigating the role of gut microbiota as extrinsic tumour factor that influences trastuzumab activity.

We found that antibiotics abrogated trastuzumab benefit in FVB mice in a microbiota dependent manner. Vancomycin and streptomycin lowered the abundance of *Clostridiales* bacteria and compromised the recruitment of immune cells in tumour microenvironment. Similar results were obtained in a model of HER2-positive BC spontaneous tumorigenesis, but not in the BALB/c model. In the attempt to improve the response to trastuzumab in FVB mice, the role of lactic acid producing bacteria was explored and an improvement in the antitumor efficacy was observed.

Patients treated with neoadjuvant trastuzumab, who achieved pCR (R) were characterized by higher abundance of *Clostridiales* bacteria and a lower representation of *Bacteroidales* as compared to those with residual disease (NR) at surgery. Transferring faecal material from R and NR into recipient mice recapitulated the response observed in patients indicating a causal role for commensal bacteria. Furthermore, the unsupervised analysis on patients gut microbiota composition identified two microbiota clusters that significantly discriminated patients according to trastuzumab benefit while no association between microbiota clusters and the HER2-enriched PAM50 molecular classification of tumour biopsies was observed. In conclusion, our data support the role of gut microbiota

composition in the therapeutic efficacy of trastuzumab independently of tumour intrinsic characteristics.

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ABBREVIATIONS

ABX	Antibiotics cocktail
ACT	Adoptive cell transplant
ADCC	Antibody-dependent cellular-mediated cytotoxicity
ADCP	Antibody-dependent cellular-mediated phagocytosis
AML	Acute myeloid leukaemia
APC	Antigen presenting cells
BC	Breast cancer
BSH	Bile salt enzyme
CDC	Complement-dependent cytotoxicity
CLL	Chronic lymphocytic leukemia
DFS	Disease free survival
DCIS	Ductal carcinoma in situ
DC	Dendritic cell
ECD	Extracellular domain
EFS	Event free survival
EGF	Epidermal growth factor
ER	Oestrogen receptor
Fc γ R	Fc γ receptor
FDA	US Food and drug administration
FFPE	Formalin-fixed paraffin-embedded
FMT	Faecal microbiota transplantation
GEP	Gene expression profile
GH	Growth hormone
GVDH	Graft versus host disease
GZMB	Granzyme B
HAT	Histone acetyltransferase
HB-EGF	Heparin-binding EGF-like growth factor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HSCT	Hematopoietic stem cell transplant
IECs	Intestinal epithelial cells
IF	Immunofluorescence
IFN- γ	Interferon γ
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry
ILC	Innate lymphoid cell
LAB	Lactic acid producing bacteria
LCIS	Lobular carcinoma in situ
LOQ	Limit of quantification
LPS	Lypopolysaccharide
mAb	Monoclonal antibody

MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MHCI/II	Major histocompatibility complex I/II
mTOR	Mammalian target of rapamycin kinase
NGR1-4	Neuregulin growth factor
NK	Natural killer cells
NLRs	Nod-like receptors
OS	Overall survival
PAMPs	Pathogens-associated molecular patterns
pCR	Pathological complete response
PCoA	Principal component analysis
PFS	Progression-free survival
PI3K	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
PPRs	Pattern recognition receptors
RLRs	RIG-1-like receptors
RTK	Tyrosine kinase receptor
SCFAs	Short chain fatty acids
STATs	Signal transducer and activator of transcription proteins
T-DM1	Ttrastuzumab emtasine
TGF α/β	Transforming growth factor α/β
Th1/2	Lymphocytes T helper 1/2
TK(I)	Tyrosine kinase inhibitor
TLR	Toll-like receptor
TMA	Trimethylamine
TMAO	Trimethylamine oxide
Treg	FOXP3 ⁺ T regulatory lymphocytes

Chapter1: INTRODUCTION

1.1 Breast Cancer

Breast cancer (BC) represents the fourth cause of cancer mortality worldwide after lung, stomach and liver cancer and occupies the second place for incidence according to the International Agency for Cancer Research (IARC) {Bray, 2018}. In Italy, BC comprises the 29% of all tumours diagnosed in women, and also represents the first cause of cancer-related death in women independently from age. Although early diagnosis contributed to the decrease of mortality, the number of newly diagnosed cases are destined to increase and more than 53.000 new BC are expected for the next years in our country {AIOM, 2018}.

1.1.1 Breast cancer classification

BC is a complex heterogeneous disease and histopathological features and molecular characteristics are both considered for its classification {Russnes, 2017}.

1.1.1.1 Histopathological classification of BC

BCs are divided into invasive and non-invasive form according to the extension of the lesion. The non-invasive forms are characterized by abnormal proliferation of cells without the spreading into the surrounding normal breast tissue and are defined as: Ductal Carcinoma In Situ (DCIS) or Lobular Carcinoma In Situ (LCIS) depending on the location of cancerous lesion within the mammary gland {Lakhani, 2012}.

The abnormal growth of cancer cells beyond the mammary duct (or lobule) define the invasive forms of BCs, which are classified into invasive breast carcinoma of “no special type” (NST) or “special subtype”. The invasive BCs of NST are the most common type of BCs and comprise all tumours without specific features of differentiation (75% of all BCs). BCs of special subtype include the invasive lobular carcinomas, tubular, cribriform, metaplastic, apocrine, mucinous, papillary and micropapillary carcinomas as well as medullary, neuroendocrine and adeno-cystic carcinomas {Lakhani, 2012}.

The histologic grade and the TNM staging system are used to evaluate the severity of the disease at diagnosis. The grading system {Elston, 1991} is based on the frequencies of tubule formation, mitotic cell number and nuclear pleomorphism and divides tumours according to a score that ranges from 1 to 3: Grade 1 (G1), well differentiated carcinomas; Grade 2 (G2), moderately differentiated carcinomas; Grade 3 (G3), poorly differentiated carcinomas. In the TNM tumour staging, the diameter of the nodules is indicated with T (0-4), with T1 that are tumours lower in size than 2 cm, T2 between 2-5 cm, T3 bigger than 5 cm and T4 is used for tumour lesions that invaded adjacent vital organs. The spreading of the disease to the lymph nodes is displayed by N (0-3), and the presence of distant site metastasis with M (0-1) {Giuliano, 2017}.

In addition to pathological feature, the analysis of molecular biomarkers are used in the routine clinical management of invasive BCs: oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 are evaluated by immunohistochemistry. The first three biomarkers represent the target of effective therapies used in the clinical practice {Marchiò, 2017 b} and they also predict the response to treatment. The expression of oestrogen receptor (ER) and the progesterone receptor (PR) predicts the efficacy of hormonal therapy {Hammond, 2010}, ER is expressed in almost 70% of BCs while 60-65% of BCs are positive to PR {Lakhani, 2012}.

The HER2 receptor is overexpressed in about 10-15% of BC and the US Food and Drug Administration (FDA)-approved Dako HerceptTest kit is used to evaluate its expression by IHC. The samples are scored according to the circumferential membrane staining, the intensity, and the frequencies of stained cells {Wolff, 2013}. Negative tumours comprise those scored as 0 (no staining) or 1+ (incomplete/faint staining on the membrane in $\leq 10\%$ of the cells). Positive tumours are scored 3+ (complete circumferential membrane staining, intense and in $>10\%$ of the cells). Tumours with score 2+ and characterized by a weak to moderate membrane staining in $>10\%$ of tumour cells, are

define equivocal {Wolff, 2018} (Fig. 1.1 A). In equivocal tumours, the IHC staining is combined with fluorescence or chromogenic in situ hybridization (FISH and CISH, respectively), to determine the copy number of the *ERBB2* gene (encoding the HER2 protein). According the updated guideline of the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) {Wolff, 2018}, the evaluation of *ERBB2* amplification comprise the use of a dual-probe system: one directed against *ERBB2* gene and one that binds to the centromere of chromosome 17 (Chromosome enumeration probe 17, CEP17). Tumours are considered positive for IHS if the dual-probe HER2/CEP17 ratio is ≥ 2 with an average HER2 copy number $<$ or ≥ 4 . Furthermore, they are positive if the single-probe average HER2 copy number ≥ 6 signals/cell. For tumours with HER2 copy number signals ≥ 6 but with a HER2/CEP17 ratio < 2 additional work-up is required and HER2 staining by IHC and ISH results should be evaluated together on section from the same tissue sample {Wolff, 2018} (Fig. 1.1 B-C).

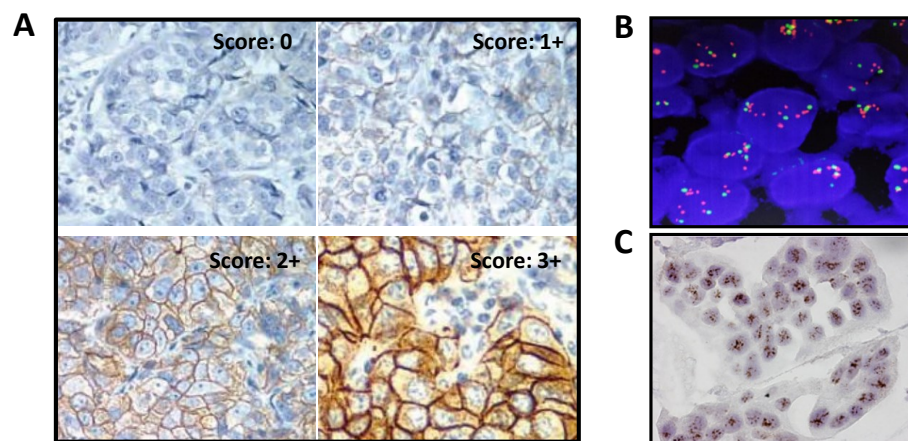


Figure 1.1 Representative images of HER2 testing in BC. **A)** IHC staining for the HER2 expression at the membrane of tumours cells (adapted from {Kamangar}). **B)** Fluorescence in situ hybridization (FISH) of a case of HER2 gene-amplified BC: HER2 amplification (red) and CEP17 (green) (adapted from {Zhao, 2017}). **C)** HER2 gene amplification by chromogenic in situ hybridization (CISH) (adapted from {Huret, 2013}).

The last prognostic marker evaluated by IHC is the Ki67, an antigen expressed by cells in active proliferation with predictive and prognostic value in ER-positive BCs treated with neoadjuvant chemotherapy {Bertucci, 2013}.

1.1.1.2 Molecular classification of BC

The evolution of genomic studies allowed the analysis of BC heterogeneity from a molecular point of view. Gene expression profile (GEP) data identified five “intrinsic subtypes” of BC {Perou, 2000 and Sorlie, 2001} classifying the breast tumours into Luminal A, Luminal B, HER2-enriched, Basal-like and Normal-like subtypes. Later in time an additional subgroup with characteristics similar to the Basal-like tumours was defined as the Claudin-low subtype {Herschkowitz, 2007}. The classification by intrinsic subtypes segregated BCs into two main clusters according to the ER status. The larger cluster comprise tumours positive for the expression of ER divided in Luminal A and Luminal B tumours. The ER negative BC comprised the HER2-enriched tumours and Basal-like subgroup including the Claudin-low tumours.

Furthermore, following a large study performed by The Cancer Genome Atlas Project (TCGA) on more than 500 of primary BC, the molecular intrinsic subtypes were refined. The comprehensive analysis at the DNA level (considering methylation pattern, chromosomal copy-number variation and somatic/germline mutation), RNA (i.e. mRNA expression and miRNA) and protein showed the existence of four main BC subgroups that recapitulated the main intrinsic subtypes found by gene expression analysis (Fig. 1.2). Claudin-low and Normal-like subtypes were not confirmed by this study due to the low number of tumours with this particular phenotype {Curtis, 2012}. Besides providing the most valuable biological information on BCs, this classification has also prognostic value (Fig. 1.3).

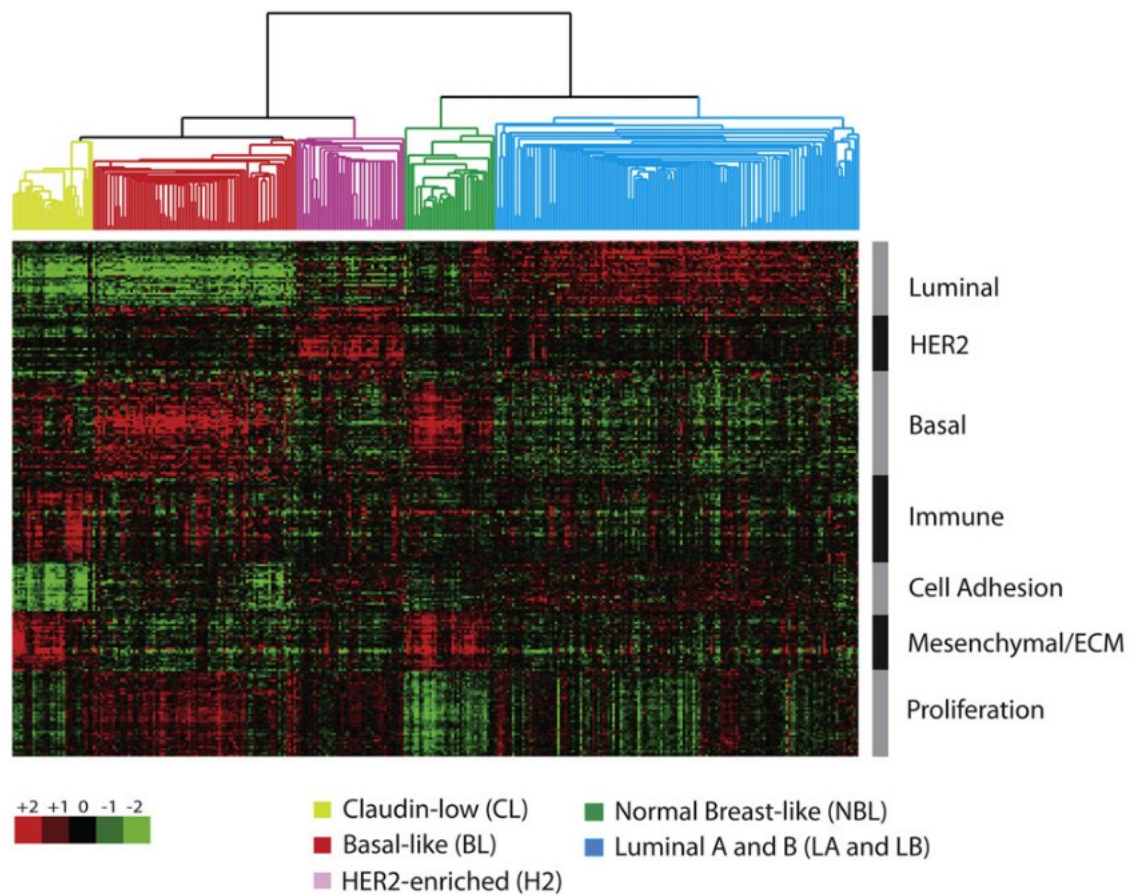


Figure 1.2 Breast cancer intrinsic subtypes. Hierarchical clustering analysis of gene expression from samples of the publicly available dataset UNC {Prat, 2011}.

Luminal A subtype

Almost 50-60% of all BCs come under the Luminal A subtype. These tumours are characterized by a low histological grade and proliferation rate, and express genes typical for the breast luminal cells {Perou, 2000} as confirmed by the positivity to the luminal cells keratins 8/18. Luminal A tumours present high expression of genes activated by the ER transcription factor such as *GATA3* and oestrogen-related *LIV-1* {Sotiriou, 2003} and at the DNA level, low number of mutation or copy-number changes were found in these tumours {Curtis, 2012}. Good prognosis and lower rate of relapse are associated with Luminal A subtype and they also benefit the most from endocrine-therapy with hormonal aromatase inhibitors or selective oestrogen receptor inhibitors (i.e. tamoxifen) and selective ER regulators such as fulvestrant.

Luminal B subtype

The Luminal B tumours comprise the 10-20% of BCs and present a more aggressive phenotype than Luminal A with a higher histological grade and proliferative index. These tumours express high levels of genes or protein related to proliferation and cell cycle (e.g. *MKI67* and *AURKA*) but they have a lower expression of several luminal genes such as *PGR* and *FOXAI*, but not *ESR1*. Genomic mutation and copy-number changes are more frequent than in Luminal A {Curtis, 2012}. Several Luminal B tumours can also have overexpression or amplification of HER2. Despite the similar expression of ER, the Luminal B subtype has a worse prognosis than Luminal A, in addition it is associated with a partial response to the endocrine therapy but benefit from neoadjuvant chemotherapy {Eroles, 2012}.

HER2-enriched subtype

This subtype includes 15-20% of all BC subtypes that are characterized by the overexpression of the ERBB2 oncogene and several genes of the HER2-pathway or the HER2 amplicon at the 17q22.24 (e.g. *GRB7*) {Perou, 2000}. These tumours present intermediate expression of luminal-related genes such as *ESR1* and *PGR*, and low expression of basal-related genes (e.g. *KRT5* and *FOXC1*), while they have the highest number of mutations across the genome among all BC subtypes, in particular common mutations were found at DNA level in *TP53* and *PIK3CA* {Curtis, 2012}. From a clinical point of view, HER2-enriched tumours are characterized by high proliferative index and high histological grade, in addition patients with HER2-enriched tumours have a poor prognosis although they benefit from neoadjuvant treatment with higher response rate than luminal tumours {Eroles, 2012}.

Basal-like subtype

The Basal-like subtype represents 10-20% of all BC, these tumours express genes typical of breast basal epithelial cells and stain positive for the basal keratins 5/6 and 17

together with EGFR at the IHC analysis. Basal tumours express high levels of proliferation-related genes (i.e. *MKI67*), intermediate HER2-related genes and very low luminal genes expression. *TP53* and *PIK3CA* are the most frequent mutated genes, and mutations in *BRCA1* are usually associated with the Basal-like subtypes {Curtis, 2012}. High histological grade, large tumour size and lymph nodes involvement together with high mitotic index are peculiar for basal tumours. Due to the absence of ER, PGR and HER2 expression they are associated with TNBC although there is a certain grade of discrepancy between the two classifications {Slamon, 1987}. Similarly to the HER2-enriched subgroup, they are characterized by worse prognosis but present high response rate to chemotherapy {Rouzier, 2015}.

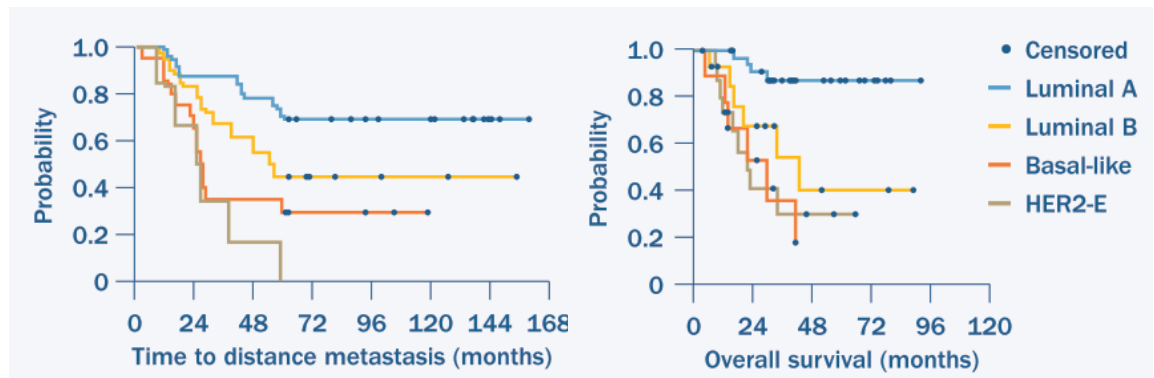


Figure 1.3 Clinical outcome of BCs according to intrinsic subtypes classification. Time to distant metastasis and overall survival curves of BC intrinsic subtypes with Claudin-low and Normal-like subtype excluded {Hoadley, 2014}.

The relevance of tumours classification in predicting patients outcome, raised the need to develop a clinical-applicable test that could define intrinsic subtype at diagnosis. In 2009, a predictor based on the expression of 50 genes was developed using microarray or real-time PCR (qRT-PCR) that was called PAM50 (Predictor Analysis of Microarray 50). This 50-gene classifier, beside reproducing with accuracy the tumour intrinsic subtypes it also maintained the prognostic and predictive value for the efficacy of anticancer treatment {Parker, 2009} observed in the previous studies.

Since 2011, the tumour intrinsic subtype-based approach was adopted for the recommendation of systemic adjuvant therapies in early BC (i.e. endocrine, chemotherapy and anti-HER2 treatment). However, due to the prohibitive costs of gene expression molecular assay, pathology-based surrogate definition of tumour intrinsic subtypes are commonly used in the clinical practice for the classification of tumours by the expression of the four biomarkers through IHC (i.e. ER, PR, HER2 and Ki67) {Goldhirsch, 2013a}:

“Luminal A-like” ER-positive; PR-positive ($\geq 20\%$); HER2-negative; Ki67 low ($< 14\%$).

“Luminal B-like” (HER2-negative) ER-positive; PR low (or negative); HER2-negative; high Ki67 ($> 14\%$) or grade.

(HER2-positive) ER-positive; HER2-overexpression/amplification;

PR any; Ki67 any.

“HER2-positive” ER and PR absence; HER2 overexpression/amplification

“Triple negative” ER, PR, HER2-negative (80% of overlap between TNBC and the intrinsic Basal-like subtype).

Although the clinicopathological surrogate is the most useful and applicable system to define the tumour subtype at the diagnosis, intrinsic biology identified by gene expression analysis provides clinical relevant information beyond the current pathology-based classification {Prat, 2015}.

1.1.2 The HER2-positive breast cancer

1.1.2.1 The human epidermal growth factor receptors family

The HER2 receptor is a type I tyrosine kinase receptor (RTK), member of the ERBB/HER super family comprising: EGFR (HER1), HER3 and HER4. These receptors are composed by a large extracellular domain (or ectodomain, ECD) dedicated to the binding of the ligands {Schlessinger, 2002}, a transmembrane region and cytoplasmic TK domain. The ligand binding region of each family member is made of four different subdomains (I-IV): domain I and III are responsible for the binding of the ligand, while domain II guides the dimerization with other receptors {Ogiso, 2002}. The ECD is usually maintained in an inactive conformation with the dimerization subdomain blocked between domain III and IV, during the interaction with the ligand the conformation changes and the subdomain II is exposed externally activating the receptor. The cytoplasmic region consists of three components: a small intracellular juxta-membrane domain followed by the kinase domain, and a C-terminal tail which is phosphorylated upon activation and contains the docking site for downstream signalling molecules {Burgess, 2003}. The HER2 receptor differs from the other family members for its extracellular region that constitutively exposes the dimerization domain. This enables the interaction with the ligand-bound receptor or with another HER2 receptor without specific EGF-related ligand (i.e. hetero-/homodimerization, respectively) {Garret, 2003}.

The HER receptors bind to three different groups of ligands: the first group includes the epidermal growth factor (EGF) that, together with the transforming growth factor α (TGF α) and amphiregulin, binds specifically to HER1; the second group of ligands includes betacellulin, the heparin-binding EGF-like growth factor (HB-EGF) and epiregulin which bind to HER1 or HER4; the last group comprises the neuregulin growth factor (NGR), in particular NRG1-2 specific for HER3 and HER4, and NRG3-4, that bind exclusively HER4 {reviewed in Hynes, 2005}. HER2 is an orphan receptor without a

physical soluble ligand and it is the preferred dimerization partner for the other HER receptors {Graus-Porta, 1997}(Fig. 1.4).

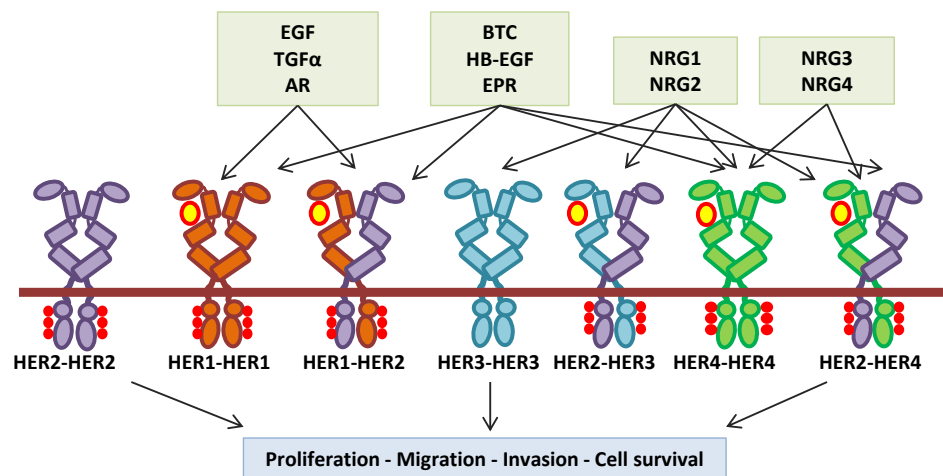


Figure 1.4 Members of the human epidermal growth factor receptor family and ligands. The structure of HER receptors consists in an extracellular ligand-binding domain, a transmembrane lipophilic domain and an intracellular tyrosine kinase (TK) domain. Ligand binding induces autophosphorylation and homo-/heterodimerization of the receptors leading to transphosphorylation with consequent activation of downstream signalling. No ligand for HER2 is known and homodimerization results in receptor activation. HER3 relies on heterodimerization for its transphosphorylation due to the absence of the TK domain. EGF (epidermal growth factor), TGFα (transforming growth factor alpha), AR (amphiregulin), BTC (betacellulin), HB-EGF (heparin-binding EGF), EPR, (epiregulin), NRGs (neuregulin) (adapted from {Hynes, 2005}).

The HER family is important for the development and all receptors cooperate to activate multiple signalling pathways that regulate proliferation, apoptosis, invasion and metastasis, angiogenesis and cell differentiation. Under normal physiological conditions, spatial and temporal expression of their ligands controls the activation of HER receptors but dysregulation in HER signalling is implicated in a wide variety of solid tumours. Amplification of *ERBB2* gene has been detected in a subset of BCs and it occurs in other human cancer (i.e. ovarian, gastro-oesophageal, bladder and salivary cancers) {Hynes, 2005}.

1.1.2.2 HER2-positive breast cancer and treatment

The HER2-positive BC subtype is characterized by aggressiveness and bad prognosis as the overexpression/amplification of the HER2 amplicon was correlated with a shorter time to relapse and a lower survival rate in women with advance BC that received only chemotherapy {Slamon, 1987}. The direct targeting of the overexpressed HER2 receptor represents the main strategy to inhibit its function in combination with chemotherapy. In 1998, the US FDA approved the use of trastuzumab (Herceptin, Genetech, Inc) in metastatic BC patients based on the positive results obtained in a large phase III study in which trastuzumab combined with chemotherapy improved patients outcome {Slamon, 2001}. Trastuzumab is a monoclonal antibody (mAb) that binds to the juxtamembrane region of HER2 and impairs the homo- or heterodimerization with other receptors resulting in the inhibition of intracellular pathway activation and tumour growth. Based on the positive results achieved in the metastatic setting, in 2006, trastuzumab was approved for the treatment of women with early stage HER2-positive BC reducing the risk of BC-related death by nearly 33% {Romond, 2005} and improving event-free survival {Piccart-Gebhart, 2005}. To date neoadjuvant and adjuvant treatment with trastuzumab, combined to chemotherapy, represent the standard of care in HER2-positive early BC {Balic, 2019}.

Over the years, new drugs have been designed to target the HER2 receptor in combination with trastuzumab: new mAbs, like pertuzumab (Parjeta, Roche), or drug-conjugated mAb (trastuzumab emtastine, T-DM1) that bind to the extracellular region have been introduced. While trastuzumab targets the juxtamembrane region (subdomain IV) of HER2, the humanized mAb pertuzumab binds to the dimerization domain (subdomain II) preventing its activation by blocking the homo- and heterodimerization of HER2 with HER1 and HER3. A different strategy considers the use of TK inhibitors (TKI) such as lapatinib and neratinib to block the activation of HER2 intracellular signalling. Lapatinib (Tyverb, Novartis) is a reversible TKI specific for HER2 and HER1 that inhibits the TK

activation by competing with the ATP for the ATP-binding pocket on the intracellular domain of HER receptors. Differently, neratinib (Nerlynx, Puma Biotechnology, Inc.) is a pan-HER inhibitor that interacts covalently with a cysteine side chain in the TK domain of HER1, HER2, and HER4 {Di Modica, 2017}.

1.1.2.3 Mechanisms of action of trastuzumab

Trastuzumab was initially developed as an inhibitor of the HER2 activation to block its intracellular signalling. As for the other family members, HER2 activates two main intracellular pathway: the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) {Yarden, 2001} together with the signal transducer and activator of transcription protein (STATs), SRC {Ishizawar, 2004} and the mammalian target of rapamycin (mTOR), which is a serine/threonine kinase activated downstream of PI3K-AKT pathway {Bjornsti, 2004}. All these molecules are involved in the regulation of proliferation and cellular growth in cancer cells. Consequently to the HER2 inhibition by trastuzumab, the induction of the Rb-related protein p130 impairs the entrance of the cell in the S-phase {Sliwkowski, 1999} resulting in the cancer cell cycle arrests in the G1 phase {Lane, HA}. The treatment with trastuzumab causes the ubiquitination of HER2 protein inducing its endocytosis and degradation, reflected by the decrease of HER2 expression on tumour cells surface observed in patients upon trastuzumab treatment {Burstein, 2003}. The binding of trastuzumab to the juxtamembrane domain, inhibits the cleavage of the HER2 ECD operated by metalloproteinases resulting in a truncated membrane-associated fragment with conserved and improved kinase activity. High serum levels of HER2 ECD correlate with poor prognosis and decrease responsiveness to endocrine therapy {Leitzel, 1995} and chemotherapy {Colomer, 2000} in patients with advance BCs.

Trastuzumab also exerts a cytotoxic activity by eliciting anti-cancer immune response. The Fc portion of the mAb is recognized by innate immune cells that express the

Fc receptor (FcγR) (e.g. natural killer (NK) cells, macrophages, neutrophils, and eosinophils) promoting an antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) against tumour cells (Fig. 1.5). Two relevant mechanisms of action for trastuzumab since its anti-tumour efficacy is lost in FcγR-deficient mice or upon FcγR inhibition in preclinical model {Clynes, 2000}. Furthermore, a loss-of-function polymorphism in the FcγR impairs trastuzumab efficacy in BC patients {Musolino, 2008 and Tamura, 2011}.

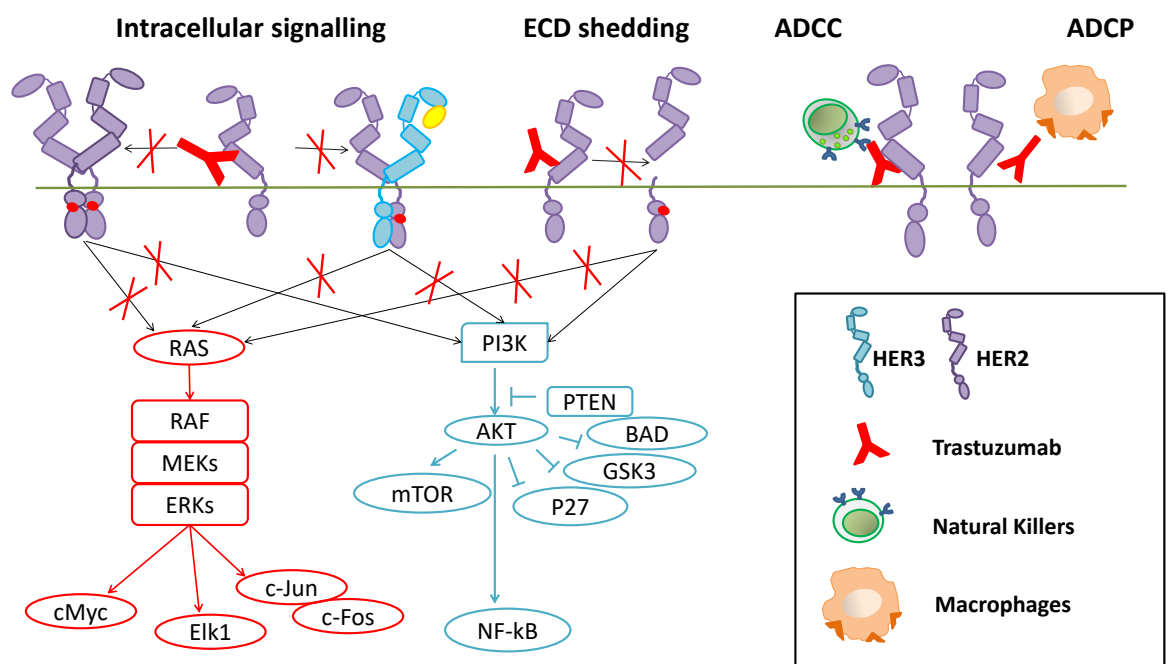


Figure 1.5 Mechanisms of action of trastuzumab. Trastuzumab inhibits the intracellular signalling downstream of HER2 homo/heterodimers and proteolytic cleavage of the HER2 ECD. By binding to the extracellular portion of HER2 engages antibody-dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) in innate immune cells (i.e., NK lymphocytes, macrophages, monocytes, and neutrophils) via the Fc portion of the antibody (adapted from {Di Modica, 2017}).

The major role in ADCC is played by NK cells that, by inducing tumour cell cytolysis {Varchetta, 2007 and Di Modica, 2016}, also increase the availability of tumour antigens that are up taken by antigen presenting cells and presented via MHC class II. Moreover, the FcγR-mediated phagocytosis by dendritic cells (DCs) and macrophages enhances antigens presentation favouring the development of tumour-specific T cell immunity. Thus, the priming of DCs (or antigen presenting cells in general, APC)

promotes the activation of CD8⁺ T cells and the polarization of CD4⁺ T cells towards a T helper 1 (Th1) phenotype. In parallel, trastuzumab-dependent NK-cell activation leads to cytokines secretion promoting the recruitment of myeloid and T cells within the tumour microenvironment {Muntasell, 2017}. By eliciting both innate and adaptive immunity, the anti-HER2 mAb exerts a vaccine-like effect that contributes to its clinical efficacy {Griguolo, 2019} (Fig. 1.6).

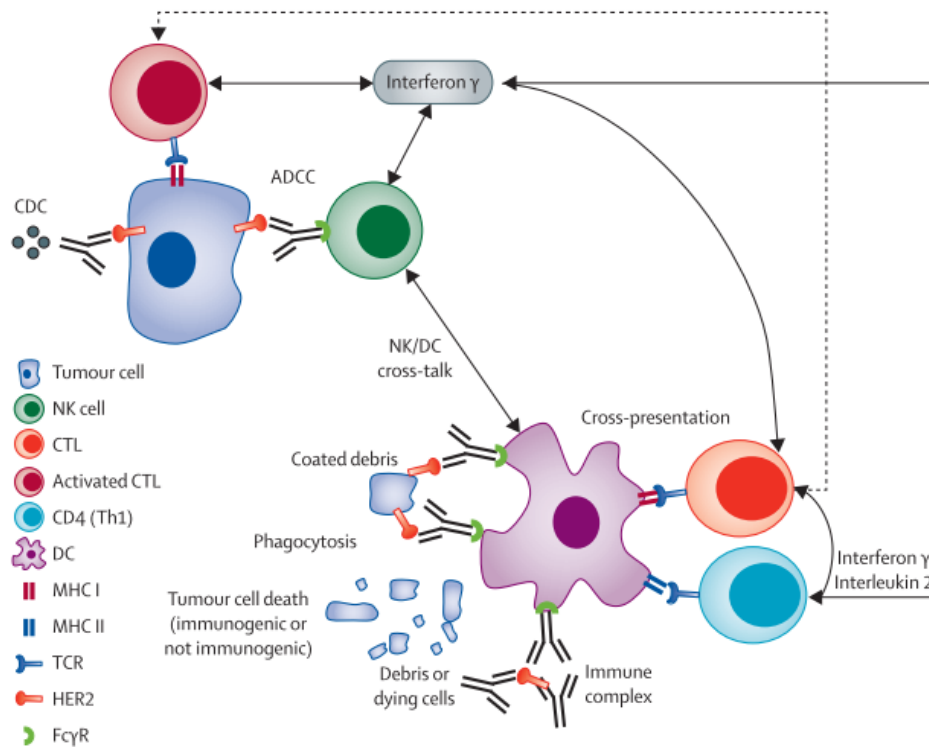


Figure 1.6 Immunostimulatory activity of trastuzumab. Trastuzumab elicits an antitumor immune response that involves the innate (i.e. natural killer cells, NK; DCs and macrophages) and adaptive (i.e. CD4⁺ and CD8⁺ T lymphocytes) immunity. The binding of trastuzumab to HER2 induced ADCC, ADCP and complement-dependent cytotoxicity (CDC). Adaptive immune cells (i.e. CD4⁺ and CD8⁺ lymphocytes) are activated by dendritic cells that recognized opsonized tumour fragments and stimulate a cytotoxic anti-tumour immunity. Antigens presented by MHC-II molecules activate CD4⁺ T-helper lymphocytes, which release interferon- γ (IFN γ), interleukin 2 (IL2) and prime CD8⁺ lymphocytes that can also recognize tumour antigens presented by MHC-I molecules directly {Bianchini, 2014}.

1.1.3 Efficacy of trastuzumab and predictive biomarkers

1.1.3.1 (Neo)adjuvant treatment with trastuzumab

The addition of trastuzumab to standard chemotherapy significantly improved the survival outcome of metastatic, advanced and early BCs. Based on the results of four large trials (HERA {Goldhirsch, 2013b}, FinHER {Joensuu, 2009}, NCCTG N9831, and the NSABP B-31 trials {Perez, 2011}) the treatment with trastuzumab is recommended for 1 year in adjuvant setting {Goldhirsch, 2011}, demonstrating improvement in DFS and mortality in patients receiving the mAb {Dahabreh, 2008}. In neoadjuvant setting, the combination of trastuzumab and chemotherapy resulted in higher pathologic complete response (pCR) rates and improved DFS in patients treated with trastuzumab compared to chemotherapy alone in phase III trials {Budzar, 2007; Gianni, 2010; Untch, 2010}. Meta-analysis on neoadjuvant studies {Valachis, 2011; von Minckwitz, 2011} concluded that the addition of trastuzumab improved the likelihood of achieving higher pCR rate. Of note, in the actual treatment of HER2-positive BC, the pCR in trastuzumab-treated patients is obtained at similar rate (46.8%) to that observed in clinical trials (40%) {Natoli, 2013}

1.1.3.2 Predictive biomarkers of trastuzumab response

Despite the improvement in the clinical outcome of HER2-positive BC patients, high grade of heterogeneity characterized the response to trastuzumab-based treatments, and, although some of the treated patients may even benefit exclusively from anti-HER2 agents without chemotherapy, many of them still do not completely respond to the treatment (i.e. less than 50% of pCR achieved in treated patients {Natoli, 2013}). For this reason, there is the need to identify accurate biomarkers that discriminate patients who actually benefit from trastuzumab treatment from who need further therapy. In this context, tumour intrinsic features and immune infiltrate have been largely investigate {Triulzi, 2016}.

Tumour intrinsic biomarkers

The heterogeneity of the HER2-positive BCs is revealed when the PAM50 molecular classifier is applied within this subgroup of tumours, in fact, only 50% of them is classified as HER2-enriched {Prat, 2014b}. These tumours are characterized by high expression of HER2-related genes and low levels of luminal BC-associated gene and the HER2-enriched phenotype is associated with higher probability of pCR rate in patients receiving trastuzumab-based treatment than other clinical HER2-positive tumours as resulted from the analysis of the major clinical trials (NOAH {Prat, 2014}, CALGB 40601 {Carey, 2016}, NeoALTTO {Fumagalli, 2017}, and CHER-LOB {Dieci, 2016}).

Coherently with the PAM50 classification, the mRNA expression levels of *ERBB2* and *ESR1* genes predict the response to adjuvant trastuzumab, with high expression of *ERBB2* and low *ESR1* associated to trastuzumab benefit {Pogue-Geile, 2013}. In the neoadjuvant setting, the levels of *ERBB2* and *ESR1* were confirmed as the most important discriminant of pCR in the NeoALTTO {Fumagalli, 2017}, GeparQuattro {Denkert, 2013} and in the TRYPHAENA trials {Schneeweiss, 2014}. In addition, the new trastuzumab risk (TRAR) predictor, which is based on the expression levels of 41 genes related to *ERBB2* and *ESR1*, is predictive of early relapse in adjuvant setting with patients classified as TRAR-low (high *ERBB2* and low *ESR1* mRNA levels) associated with better prognosis {Triulzi, 2015}. In the neoadjuvant setting, the TRAR signature predicts pCR in the NeoALTTO study {Di Cosimo, 2019} confirming the role of HER2 and ER expression in trastuzumab benefit.

The higher sensitivity of HER2-enriched tumours to anti-HER2 agents was confirmed in the PAMELA trials {Llombart-Cussac, 2017}, in which higher percentage of pCR was observed in HER2-enriched tumours compared to other subtypes treated with neoadjuvant dual blockade with trastuzumab and lapatinib without chemotherapy. However, the fact that only half of HER2-enriched tumours (41%) achieved pCR, indicate

that further improvement are needed to accurately predict whether one will benefit from anti-HER2 treatment.

Immune biomarkers

Based on the relevance of immune cells in trastuzumab activity the predictive or prognostic potential of the tumour immune contexture was analysed. The tumour infiltrating leukocytes (TILs) include different cells type of mononuclear leukocytes such as macrophages, DCs, mast cells, NK cells, naïve and memory lymphocytes, B cells and effector T cells (comprising different subset of T cells such as Th1, Th2, Th17, regulatory T cells (T reg) and cytotoxic T cells). These immune population can be divided according to their role in cancer development and progression, CD8⁺ T cells, CD4⁺ Th1 and NK cells are generally considered to favour tumour suppressive responses, while CD4⁺ Th2, FOXP3⁺ Treg and immature DCs might have a pro-tumorigenic role {Salgado, 2015}. TILs are easily quantified through the hematoxylin/eosin staining of tumour biopsies considering both intratumoral and stromal leukocytes {Loi S, 2014}, BCs highly infiltrated (>50% TILs) are defined as lymphocytes predominant BCs (LPBC). According to recent guidelines, TILs should be scored as continuous parameters to better represent the continuity of immune response with >60% of TILs set as cut off for highly infiltrated tumours {Salgado, 2015a; Dieci, 2018}. The prognostic value of TILs was described for the first time by Denkert and collaborators {Derkert, 2010} that demonstrated the prediction of pCR achievement by TILs abundance in BCs patients treated with neoadjuvant chemotherapy. In the HER2-positive BC the high infiltration of TILs was consistently associated with pCR in neoadjuvant trials such as NeoALTTO {Salgado, 2015b} CHER-LOB {Dieci, 2016} and NeoSphere {Bianchini, 2015}.

The immune status of patients can be also characterized by genomic signature. Perez and collaborators developed a signature consisting of 14 immune-related genes that discriminate tumours as immune response enriched (IRE) and non-enriched (NIRE)

{Perez, 2016}. Patients classified as IRE had tumours enriched in genes related to T and B cell responses, chemokine signalling and inflammation, these patients had a better DFS when treated with trastuzumab. In neoadjuvant setting, the expression of this signature and other interferon-related gene, along with STAT1 but not MHCI metagenes {Bianchini, 2015}, highly correlated with pCR {Fumagalli, 2017}. Although the high predictive value of TILs and immune signatures in the neoadjuvant treatment, the prediction value of TILs in adjuvant setting is controversial and required further investigation {Loi, 2014; Perez, 2016}.

New emerging approaches to identify novel predictive biomarkers exploit the analysis of samples pre- and post-treatment, as for instance immune characterization in residual disease upon neoadjuvant treatment {Dieci, 2018}. In addition, the modulation of immune gene upon single-dose of trastuzumab revealed that the increment in the levels of MHCII metagenes {Triulzi, 2018b} and the expression of CD4⁺ T cell- related metagene {Varadan, 2016} were predictive of response to therapy.

When the impact of tumour-related and immune-related features were analysed to understand the response to neoadjuvant chemotherapy plus anti-HER2 agents, immune signature added significant prediction of pCR beyond PAM50 classification {Dieci, 2016} supporting the need for the integration of such predictive biomarkers to increase the accuracy.

1.1.3.3 Sources of tumour heterogeneity and immune infiltrate modulation

Understanding which factors modulate immune infiltrate can help in the development of new treatment strategies and add further information to the already used predictive biomarkers. The most evident driver of heterogeneity in HER2⁺ BCs is the ER status, many immune parameters are inversely correlated with ER expression {Chung, 2017}. Oestrogenic signalling interacts with immune activity, oestrogen might modulate susceptibility of cells to NK-mediated ADCC by up-modulating MHCI transcription

{Hamada, 1989}. The immunosuppressive activity of myeloid cells can be enhanced by oestrogen {Svoronos, 2017}, thus menopausal status of patients should be considered in the analysis of tumour immune microenvironment. The immune infiltrate is also dictated by high mutation burden corresponding to a greater amount of neoantigen found in HER2 enriched tumours {reviewed in Griguolo, 2019} and infiltration of HER2-positive tumours by immune can also be associated with oncogene activation {Triulzi, 2018a}.

Besides tumour molecular features and genetic (or epigenetic) alteration, life style, physical activity, sedentary behaviour, and diet have a determinant role in the development and progression of BC. While dietary fibre retard carcinogenesis increasing tumour infiltration by CD8⁺ cells, obesity (or metabolic obesity state) induces a systemic inflammatory status (i.e. increasing the levels of IL-6 and aromatase) but results in an immunosuppressive tumour microenvironment with BC from obese patients characterized by high levels of immunosuppressive macrophages {Howe, 2013}. Furthermore, dietary changes or antibiotic use may result in shift of the gut microbiota communities with a consequent impact on host immunity that may negatively affect the immune infiltrate of BC {Kroemer, 2015} (Fig. 1.7).

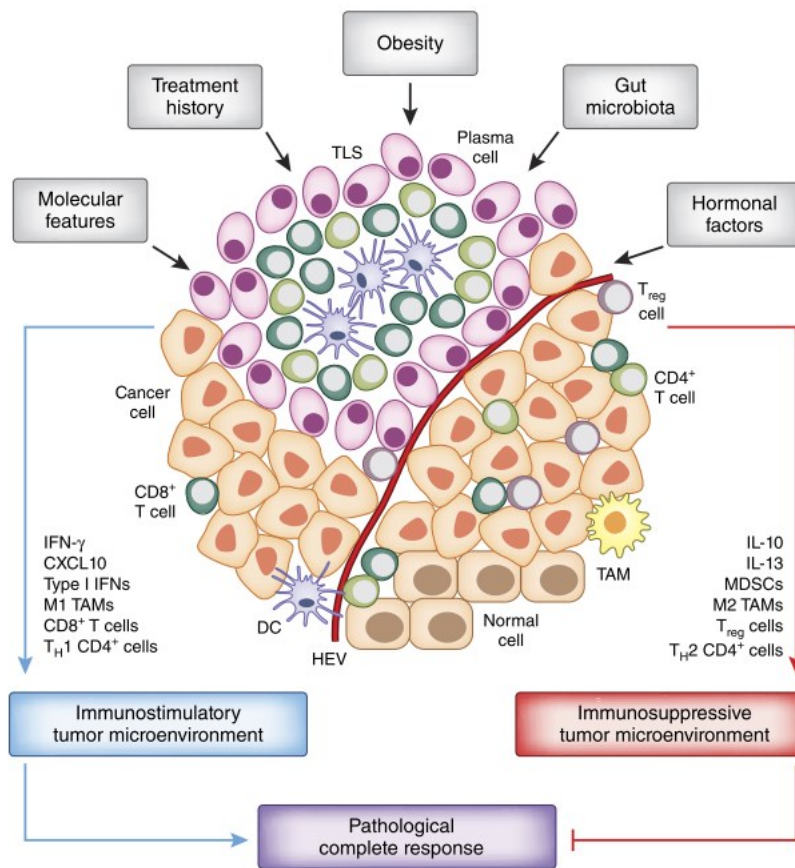


Figure 1.7 Factors that condition tumour immune microenvironment. Tumour molecular characteristics and external factors such as treatment history, obesity, gut microbiota and hormonal factors determine the abundance and functional state of the tumour immune infiltrate inducing an immunostimulatory or immunosuppressive tumour microenvironment {Kroemer, 2015}.

1.2 The gut microbiota

The human microbiota comprises all the communities of commensal, symbiotic and pathogenic microorganisms that inhabit the body. The majority of them colonize the surface of the gut, skin and oral cavity and it is estimated that the human body is composed by 3×10^{13} eukaryotic cells and 3.9×10^{13} colonizing microorganisms {Sender, 2016}. Overall, the gut of each individual is colonized by more than 160 species of the 1200 that have been identified for a total biomass of 1.5-2 kg dominated by anaerobic Bacteria, Archaea, Eukarya and Viruses {HMP Consortium, 2012}.

1.2.1 Methods to study the gut microbiota composition

The possibility to study the gut microbiota characteristics and functions notably increased in parallel with the development of new techniques for its analysis. The strategies that can be used to study the microbiota are divided into culture-based or culture-independent approaches. Bacterial culture allows cheap, but labour intensive, identification of bacterial species within the intestine, providing functional microbiome analyses. Although this methodology significantly improved in the last decade increasing the number of culturable bacteria from 30 to 70 % of total gut communities, it still gives a limited view of the microbial diversity restricted to those culturable microbiota members {Zuo, 2019; Ito, 2019}.

A more defined ‘portrait’ of the intestinal microbiota, in terms of composition, has become possible through the advancement of culture-independent techniques comprising all those ‘omics’ disciplines, that consider the study of DNA, RNA, protein and metabolites:

- Metagenomics (DNA): the study of the metagenome; it includes amplicon-based studies focused on marker genes such as 16S ribosomal RNA (16S rRNA), or shotgun sequencing analyses.

- Metatranscriptomics (RNA): the study of the metatranscriptome; it analyses the collective repertoire of mRNA within a biological sample
- Proteomics (proteins) and metabolomics (metabolites): the study of metaproteome and metabolome; it examines protein and metabolites derived from the bacteria activity.

The experimental questions and the type of sample in use should guide the researcher for the decision on which method to use. In the next future, methods for integrating all metagenomics and metabolomics information, that are now under development, will allow to have a more accurate ‘snapshot’ of the composition and function of the gut microbiota {Knight, 2018}.

1.2.1.1 Microbiome metagenomic and metatranscriptomic analysis

The majority of the studies investigating differences within the complex bacterial communities of the gut, relies on DNA- and RNA-based approaches that allow metagenomic taxonomy and functional assignment.

Marker gene sequencing

Sequencing represent the gold standard for taxonomic identification of bacteria and most of the studies are based on amplicon analyses in which a specific piece of DNA is amplified. In these analyses, PCR primers are used to amplify all the variants that occur between the highly conserved regions of a specific gene: the 16S ribosomal RNA (16S rRNA) for Bacteria and Archea and the internal transcribed spacer for Fungi. The 16S rRNA is a molecule of RNA contained in the 30S subunit of the bacterial ribosome designated to the initiation and extension of protein synthesis. It is highly conserved between bacterial species but it also contains variable regions that allow phylogenetic signals for taxonomic classification (Fig. 1.8). The taxonomic names are generally assigned by matching the DNA sequences to reference databases, three of the most characterized and frequently used databases are Greengenes {McDonald, 2012}, the Ribosomal Database Project (RDP) {Cole, 2014} and Silva {Pruesse, 2007}. This method

is suitable and works well for samples contaminated by host DNA, such as tissue and low-biomass samples. In general, genus-level resolution is possible for most bacterial taxa, but, with 16S RNA gene sequencing, species resolution is difficult {Bokulich, 2018}.

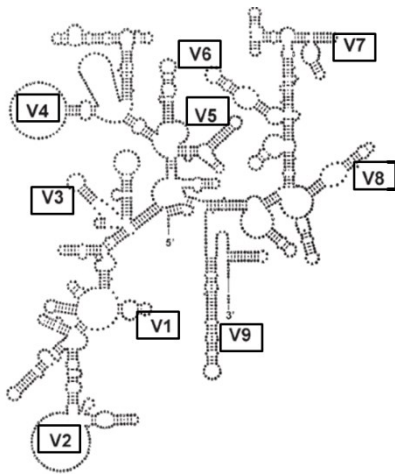


Figure 1.8 Ribosomal 16S RNA gene hypervariable regions. Nine hypervariable regions (V1–V9) are indicated on the secondary structure of 16S rRNA. These regions are usually sequenced for taxonomic classification of the bacteria (adapted from {Erlandsen, 2005}).

Whole metagenome ‘shotgun’ analysis

Metagenomic sequencing considers all microbial genomes within a sample providing more detailed information and taxonomic resolution than single gene sequencing, although more expensive to prepare and difficult to analyse. ‘Shotgun sequencing’ fragments all the DNA from a sample into small pieces, sequences these fragments and tries to reconstitute the overlapping sequences into a view of the microbiome. Given adequate sequencing depth (the number of sequencing reads per sample), taxonomic resolution reaches species and even strain level {Scholz, 2016} by assembling the whole microbial genome obtained from short DNA sequence reads thanks to the use of reference database {Mukherjee, 2017}. Furthermore, with the metagenomic sequencing profile, the functional capability of an entire community can be reconstructed at gene level {Abubucker, 2012}. However, an important limitation to shotgun metagenomics is that all the DNA will be sequenced including the human DNA which constitutes a relevant source of contamination from the host.

Metatranscriptome analysis

Metatranscriptomics is different from both 16S and metagenomic sequencing, these two methods analyze the DNA within a sample regardless of cell viability or activity,

while metatranscriptomics offers a unique insight into the microbiota revealing the microbial responses to niche perturbation. This methodology is very challenging since most bacterial transcript only last a few minutes {Laalami, 2013} and it is biased towards microorganisms with high rate of transcription {Giannoukos, 2012}. In addition, samples should be carefully preserved to avoid RNA degradation {Knight, 2018}. Metatranscriptomics and metaproteomics, the analysis of the protein content in a sample by mass spectrometry, are two emerging technologies that allow a functional study of the microbiome. Despite the interesting potential of these disciplines, further improvements are required for the complex interpretation of the results which is also complicated by the fact that no correlation between mRNA expression and protein at the all-community level has been found in the few studies performed so far {Allaband, 2019}.

The pros and cons of genomic analyses for evaluating microbial communities have been summarized in the Table 1.1.

Table 1.1 Genomic approaches to study microbial communities (adapted from {Knight, 2018}).

Methods	Pros	Cons
Marker gene (16S rRNA) analysis	<ul style="list-style-type: none"> • Fast and inexpensive sample preparation and analysis • Useful in low-biomass and highly host-contaminated samples • Large public data sets for comparison 	<ul style="list-style-type: none"> • No live/dead or active discrimination • Amplification bias • Bias introduced by the choice of primers and variable regions • Resolution limited to genus level • No functional information
Shotgun metagenome analysis	<ul style="list-style-type: none"> • Microbial taxonomic and phylogenetic identity until strain resolution for known organisms • Relative abundance of bacteria functional gene • No PCR-related biases • Does not assume knowledge of microbial community 	<ul style="list-style-type: none"> • Expensive and laborious analyses • Contamination from host-derived DNA • No live/dead active discrimination • Possibility of assembly artifacts in population-averaged microbial genomes
Metatranscriptome analysis	<ul style="list-style-type: none"> • Estimation of actively transcribing microorganisms when paired with marker gene analysis • Capture of the dynamic intra-individual variation and response to intervention and event exposure 	<ul style="list-style-type: none"> • Most expensive, laborious and complex sample preparation • Host mRNA contamination and need to remove rRNA • Careful sample collection and storage • Paired DNA sequencing to decouple transcription rate from bacterial abundance changes required

1.2.1.2 The study of the metabolome

Metabolomics is emerging in the study of gut microbiota functions and activities, it includes the nonprotein small molecules that are a product of bacterial metabolisms. The metabolome can be studied through two different approaches that are defined targeted and untargeted metabolomics. In the first case targeted metabolites are analysed according to the availability of reference standard while untargeted metabolomics allows for molecules discovery although it is less sensitive and quantitative {Melnik, 2017}. To study the metabolome, gas chromatography or liquid chromatography are used to separate metabolites before mass spectrometry analysis, and it is important to consider whether the molecules of interest will be captured or not when choosing which platform to use {Allaband, 2019}. Similarly to metatranscriptomics and metaproteomics, the study of the metabolome is emerging as a strategy to study the functions of the gut microbiota and it is usually coupled with 16S rRNA or shotgun sequencing in order to provide relevant information on both composition and activity of the intestinal bacteria.

1.2.2 Composition of the gut microbiota

The intestinal microbiota composition consists of a large number of microbial species. *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* are the dominant phyla that inhabit the gut with specific distribution varying along the gastrointestinal track (Fig. 1.9). The bacterial abundance increases from the proximal to the distal colon according to physiological variation of chemicals, pH, nutrients and mucus along the small intestine, caecum, and colon. The small intestine is structured in villi and crypts with a thin mucus layer that favours the colonization of adherent bacteria (e.g. segmented filamentous bacteria, SFB), whereas the colon presents an outer mucus layer that constitutes the optimal habitat for mucin-degrading bacteria and a dense inner layer that is scarcely colonized except for restricted communities of bacteria. *Lactobacillaceae* and *Enterobacteriaceae* families are the most abundant in the small intestine while *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae*

better tolerate the increment of pH from proximal to distal colon (Fig. 1.9) {Donaldson, 2016}. The competition for similar environmental conditions and nutrients limits the outgrowth of bacteria favouring the equilibrium within and among bacterial species {Kommineni, 2015}.

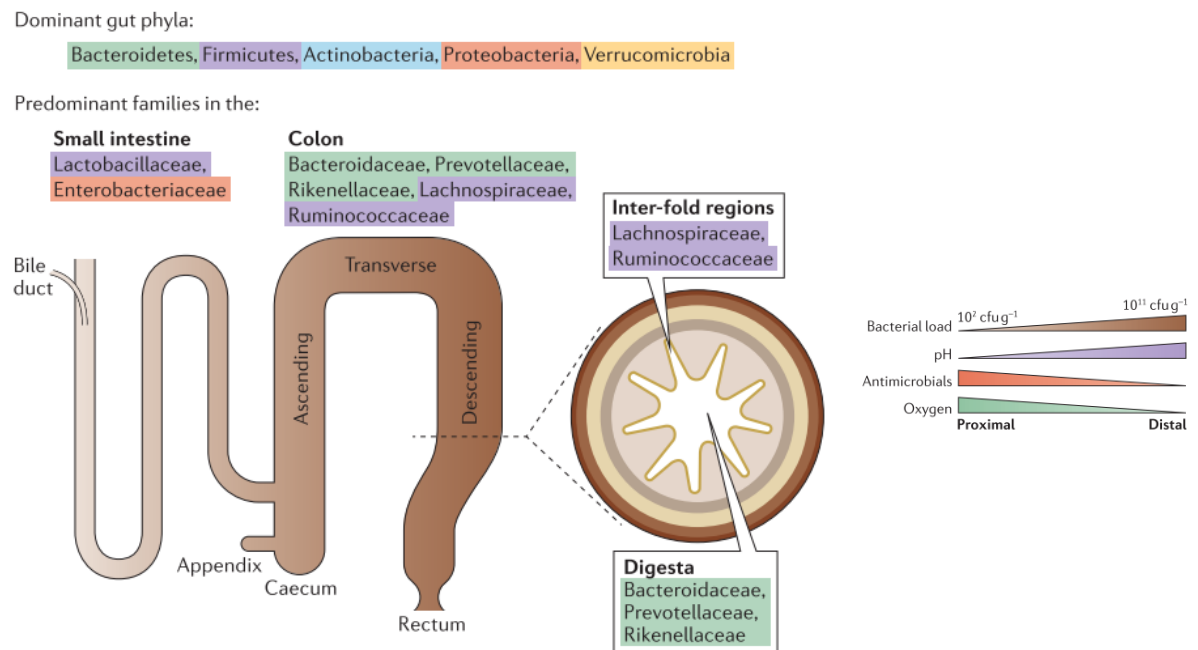


Figure 1.9 Gut microbiota composition in the human lower gastrointestinal tract. The digestive tract of human is inhabited by *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* whose distribution within the gut vary according to the different levels of oxygen, nutrients, pH and mucus layer (adapted from {Donaldson, 2016}).

1.2.3 Gut microbiota and immune system

The intestinal microbiota regulates many local and systemic function of the host including the development and regulation of the immune system, especially with respect to the intestinal mucosal immunity where it favours the maturation of immune cells in Payer's patches, mesenteric lymph nodes and the development of lymphoid follicles in the lamina propria {reviewed in Rooks, 2016}. Systemically, commensal bacteria regulate myelopoiesis in the bone marrow {Gorjifard, 2016} via circulating bacteria-derived molecules (lipopolysaccharide, LPS; peptidoglycan, or flagellin) and bacterial metabolites (e.g. short chain fatty acids, SCFAs).

1.2.3.1 Microbial messengers

Microbial-derived signalling molecules

The host immune cells discriminate pathogens from commensals recognizing conserved molecular patterns that are divided into microbe-associated molecular patterns (MAMPs, expressed by commensals bacteria), and pathogens-associated molecular patterns (PAMPs, typical of microbial pathogens). Bacteria presenting MAMPs such as LPS, peptidoglycan, flagellin or other structural components are recognized by pattern-recognition receptors (PRRs) that include Toll-like receptors (TLRs), Nod-like receptors (NLRs) or RIG-1-like receptors (RLRs) that are expressed on epithelial and immune cells (i.e. monocytes, macrophages, NK cells and T lymphocytes) {reviewed in Medzhitov, 2007; and Negi, 2019}. Although the majority of the interactions between gut bacteria and host occur at the level of the intestinal epithelial barrier {Coleman, 2018}, a wide variety of ligands for receptors expressed on immune cells can enter the circulatory system and signal at distance. These ligands include LPS, which signals through TLR4; caspase-4/5/11, an intracellular sensor; and polysaccharide A from *Bacteroides fragilis* that binds to TLR2 {Fischbach, 2016}. Regulation of host immune system also happens by shaping metabolic pathways of immune cells like the catabolism of tryptophan into the metabolite indole-3-aldehyde, a ligand of the aryl hydrocarbon receptor (AHR), which is an important immune modulator in innate lymphoid cells {Zelante, 2013}.

Bile acids

Bile acids are synthesized from cholesterol in the liver, and are released in the small intestine to enhance digestion and absorption of lipids and fat-soluble vitamins. Their reabsorption occurs in the ileum and colon, and only a small part (<5%) is secreted in faeces. Bacteria present in the gastrointestinal tract (including *Bifidobacterium*, *Lactobacillus*, *Clostridium* and *Bacteroides*) possess bile salt hydrolase enzymes (BSH) and initiate bile acid metabolisms by deconjugating the glycine or taurine from the sterol

core of the primary bile acid core (i.e. cholic acid and chenodeoxycholic acid). This mechanism prevents their reabsorption in the ileum and promotes their entrance in the large intestine where they undergo to microbiota-encoded conversion to secondary bile acids by bacteria that possess hydroxysteroid dehydrogenases {Ridlon, 2014}. Deconjugated primary and secondary bile acids have effects on the host and regulate immunity by signalling on the farnesoid X receptor (FXR)- α expressed by macrophages whose activation promotes anti-inflammatory response {Vavassori, 2009}. The proinflammatory response in monocytes and macrophages is also dampened by strong activation of TGR5 induced by secondary bile acids deoxycholic and lithocholic secondary bile acids {Perino, 2014; Pols, 2017} (Fig 1.10).

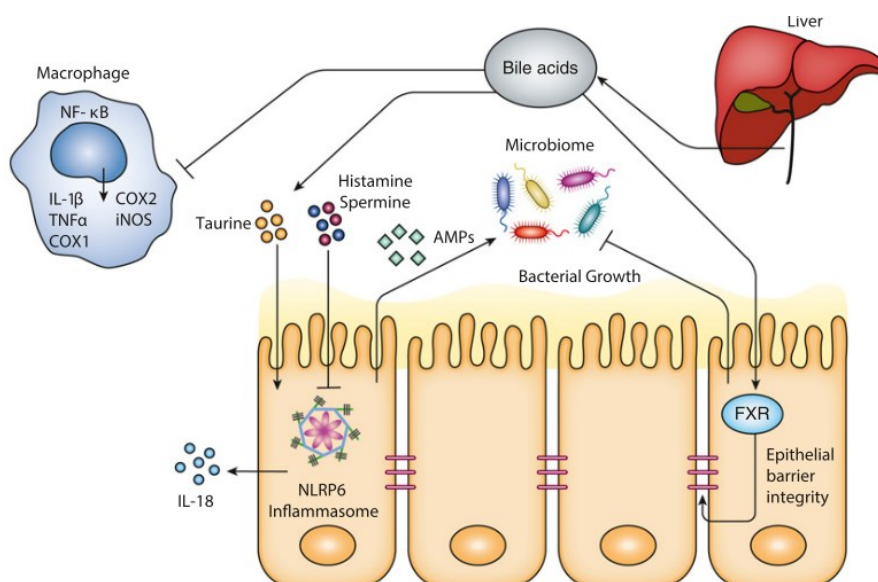


Figure 1.10 Immune regulation by bile acids. Bile acids, produced in the liver, regulate barrier integrity and commensal community. They also exert immune regulatory function by controlling the proinflammatory response in immune cells {Blacher, 2017}.

Short chain fatty acids (SCFAs)

SCFAs represent the major fermentation products generated from bacterial fermentation of dietary fibre and comprise butyrate, propionate and acetate. In the human gastro intestinal tract the highest SCFAs concentration is found in colon at a molar ratio of approximately 60:20:20 for acetate:propionate:butyrate {reviewed in Parada Venegas, 2019}. They are usually used by intestinal epithelial cells (IECs) as source of energy but

can also act as regulators of the immune system. These metabolites modulate different aspects of IECs and leukocyte development and function through activation of G protein-coupled receptors (GPR43, GPR41, GPR109a and Olfr78), inhibition of histone deacetylases (HDAC) and stimulation of histone acetyltransferase (HAT) activity. These receptors show distinct patterns of expression and have been partially associated with the effects of the SCFAs on leukocytes and intestinal epithelial cells. SCFAs enter the cells through passive diffusion across the plasma membrane or via transporters such as Slc16a1 and Slc5a8. (Fig. 1.11) {reviewed in Corrêa-Oliveira, 2016}.

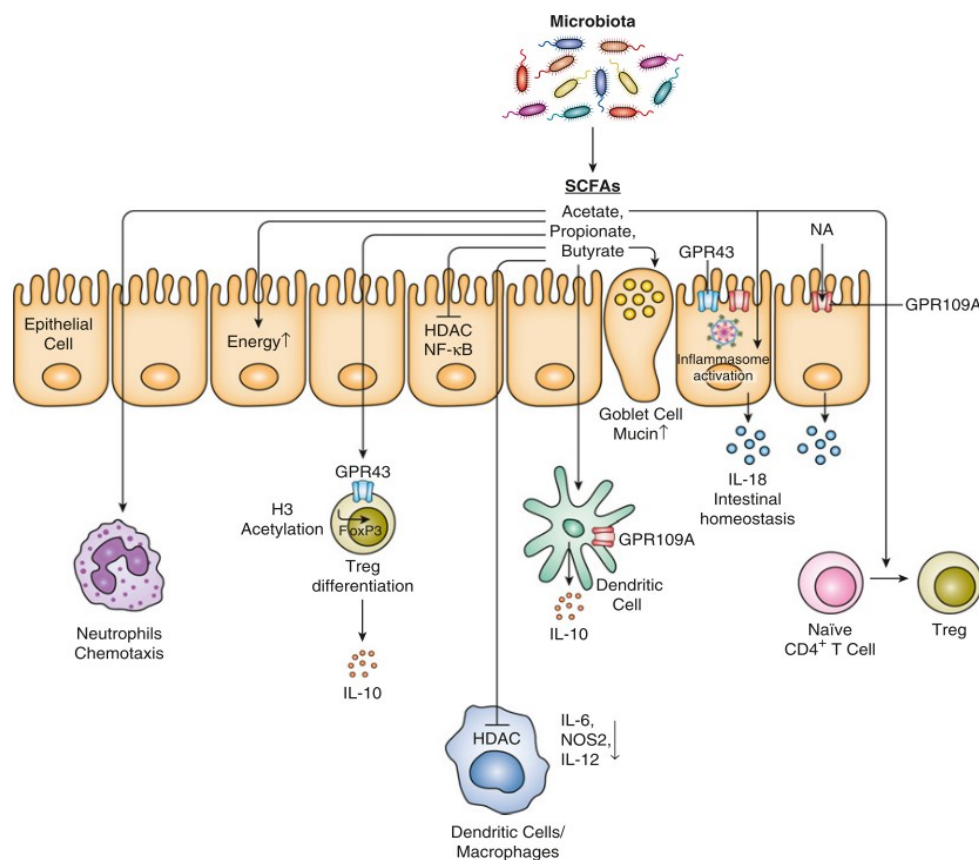


Figure 1.11 Modulation of intestinal and systemic immune cells by SCFAs. SCFAs are uptake by IECs through passive and active mechanisms (i.e GPR43, GPR41, GPR109a and Olfr78 or Slc16a1 and Slc5a8 transporters), when uptake they can be used as a source of energy or exert immune regulatory function on DCs, macrophages, neutrophils and T lymphocytes {Blacher, 2017}.

1.2.3.2 Regulation of host immunity

Innate and adaptive immune populations are equally influenced by commensal bacteria in a context-/ cellular- and timing-dependent regulation.

Innate immune cells

The sensing of microbial molecules by TLRs and NLRs influences the transcriptional programming of innate immune cells regulating the proinflammatory response in myeloid {Chang, 2014} and lymphoid cells {Gury-BenAri, 2016}. The communication between microbiota and innate immune cells rely also on metabolites and secondary bile acids {Negi, 2019}, it can differ according to the cells type: innate lymphoid cells (ILCs) count on tryptophan metabolites while myeloid cells are sensitive to SCFAs {Chang, 2014}.

Mononuclear phagocytes (i.e. monocytes, macrophages and dendritic cells) are important intermediates for the cross-talk between microbiota and host immune system. Monocytes, by sensing gut microbiota variation, regulate the trafficking of myeloid cells, in particular macrophages, in the intestinal mucosae {Sorg, 2010}. In addition, the activity of mucosal macrophages is regulated by butyrate towards an M2/tolerogenic phenotype {Scott, 2018} while gut derived metabolites trigger type I interferon signalling and potentiate their phagocytic activity in distal macrophages {Steed, 2017}. In the intestine, dendritic cells activity in promoting the downstream activation of T and B cells, is regulated by the signalling through TLR2 via MyD88, promoted by bacteria like *Bacteroides fragilis* {Surana, 2014} and by microbial product such as succinate, acetate and butyrate that bind respectively to the GPR91 or GPR43 {reviewed in Skelly, 2019}. SCFAs, in particular butyrate, seem to inhibit DCs maturation {Andrade-Oliveira, 2015} and reduce secretion of IL12p40 and IFN γ {Liu, 2012} *in vitro* acting as inhibitor of HDACs activity via the SLC5a8 butyrate transporter {Gurav, 2015}.

Different composition of gut microbiota is correlated with variation in the maturation of neutrophils {Danmlund, 2016} while their activity can be regulated by SCFAs via GPR43 activation and consequent modulation of pro- {Mirmonserf, 2012} and anti-inflammatory {Vinolo, 2011} cytokines production.

Commensal microorganisms also regulate the maturation and the acquisition of the tissue-specific function of ILCs {Minton, 2019}, a family of innate immune cells with cytotoxic (NK cells) and non-cytotoxic function (ILC1, ILC2 and ILC3).

Adaptive immune cells

The adaptive immune system is influenced by the bacterial composition in the gut in many aspects. The microbiota is a strong inducer of proinflammatory Th17 and regulatory T cells in the intestine {Ohnmacht, 2015} and this is directly regulated by specific bacterial species such as the attachment of SFB to the IECs promoting the maturation of Th17 and *Bacteroides fragilis* that, through its polysaccharide A, influences the balance between Th1 and Th2 cells and also stimulates Treg {Atarashi, 2013}. Evidence demonstrated the relevance of TLRs for the regulation of adaptive immune cells function and development. Through the interaction with TLRs, microbial stimuli induce the polarization of naïve T CD4⁺ cells into pro- or anti-inflammatory subset (such as Th1, Th2, Th17 and Treg) {Valentini, 2014} and induce CD8⁺ T cells activation {Tabiasco, 2006}. Furthermore, the SCFAs function as inhibitors of HDACs promotes the extra-thymic peripheral polarization of Treg {Arpaia, 2013} and plays a role in the balance between Th1 and Th17 polarization of T cells {Park, 2015}.

The microbiome influences the accumulation of intestinal plasma cells that produce secretory IgA, while some species promotes the production of sIgA {Hapfelmeier, 2010} some members can degrade and inhibit their production by modulating plasma cells activity {Moon, 2015} and shaping mucosal immunity.

1.2.4 Gut microbiota in health and disease

The gut microbiota influences many local and systemic functions of the host including metabolism, inflammation and the development and regulation of most organ systems. The colonization of the gastrointestinal mucosae contributes to the intestinal homeostasis by strengthening the gut barrier and mucosal immunity. Furthermore, the gut

microbiota participates to the absorption of nutrient from diet contributing to the 6-10 % of daily calories intake derived from the digestion of plant carbohydrates and fibre otherwise indigestible by human {Bergman, 1990} and sustain the production of SCFAs and the synthesis of vitamins (e.g. B2; B12; K; and folic acid) molecules that can be exploited as messengers to intestinal mucosae and distant organs or tissues in the host (liver, adipose tissue, brain, cardiovascular system, lung, hormone and immune system) {Schroeder, 2016}.

In normal conditions, the colonization of pathogens is prevented by production of antimicrobial substances, occupation of ecological niches, nutrients competition and enhancement of IgA secretion by mucosal B cells, however significant changes in the gut microbiota composition (i.e. dysbiosis) can occur and negatively affect local and systemic homeostasis leading to a large variety of disorders (Fig. 1.12).

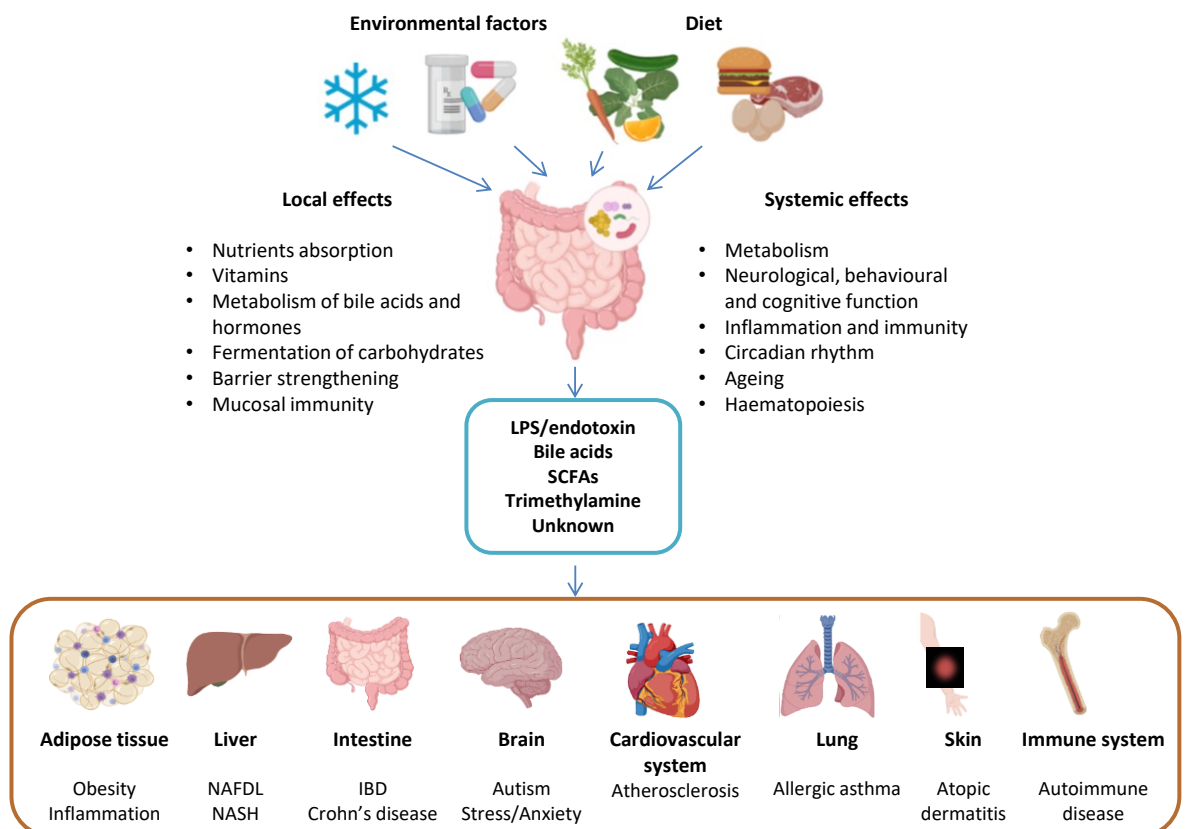


Figure 1.12 Effects of gut microbiota on host physiology. The gut microbiota communicates with the host converting environmental signalling into metabolites which can signal locally or systemically to different organs and tissues in health and disease (adapted from {Schroeder, 2016 and Roy, 2017}).

Communication between commensals bacterial and host adipose tissue, mediated by FXR- α and TGR5 receptors signalling, regulates various aspects of thermogenesis {reviewed in Schroeder, 2016} in healthy individuals. Obesity and undernourishment are influenced by the intestinal microbiota and a reduced microbial diversity or a reduced activity of the axis growth hormone (GH)-insulin-like growth factor-1 (IGF-1) are associated to these metabolic conditions respectively {Schroeder, 2016}. In liver of obese individuals, the translocation of bacterial molecules trigger hepatotoxicity and progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) via the activation of TLRs signalling {Henao-Mejia, 2012}. Cardiovascular disease, and in particular the development of atherosclerosis, are associated to the gut microbiota composition due to the action of microbial enzymes such as trimethylamine (TMA) lyases which convert the choline and carnitine, derived from diet, into TMA further oxidised to trimethylamine oxide (TMAO), a toxic compound involved in the pathogenesis of atherosclerosis {Wang, 2011; Matsuzawa, 2019}. Several autoimmune diseases have been described to be influenced by commensal bacteria {Ho, 2015}, for instance type I diabetes or juvenile diabetes are characterized by imbalanced in the ratio between *Bacteroidetes* and *Firmicutes* and a reduced number of butyrate producing bacteria {de Goffau, 2014}. Similarly, in inflammatory bowel disease (IBD), a gastrointestinal disorder partly due to autoimmune deregulation and characterized by recurrent inflammation of the intestinal mucosal, dysbiosis and decreased microbial diversity, is linked to both the two phenotypes of IBD: Crohn's disease {Hedin, 2014} and ulcerative colitis {Walujkar, 2014}. Other autoimmune condition such as allergies {West, 2014} and rheumatoid arthritis {Zhang, 2015} have been linked to the gut microbiota status, in particular low microbial diversity has been observed to precede allergic disease {West, 2014}.

Recently, evidence for gut microbiota influence on the development of the brain structure and response to stress has emerged. Intestinal microbes communicate with the

brain through microbial metabolites able to cross the blood brain barrier {Erny, 2015} or via the vagus nerve {Bravo, 2011; Li, 2018}. The microbiota-brain axis was described to have a role in both the development and treatment of psychiatric stress-related conditions such as depression and anxiety {CrumeYrolle-Arias, 2014}. Furthermore, association with an altered abundance of *Firmicutes*, *Clostridiales* and *Bifidobacterium* species, compared to control subjects, was found in children affected of autism, a developmental disorder marked by impaired interaction and restricted/repetitive behaviour, highlighting the possible connection between cognitive impairment and commensal bacteria {Meyer, 2011}.

1.2.5 Gut microbiota, cancer and cancer therapy

1.2.5.1 Bacteria-related tumorigenesis

Certain bacteria species are causally linked or associated with cancer development: *Helicobacter pylori* is classified by the World Health Organization (WHO) as a class I carcinogens due to its genotoxic effects that promote gastric cancer {Ishaq, 2015}. *Salmonella typhi* and *Helicobacter spp* along with *Fusobacterium nucleatum* participate to the development of biliary {Mima, 2017} and colorectal cancer respectively {Kostic, 2013}. The microbiota-related tumorigenesis can also be a secondary effect of local, or systemic, chronic inflammation caused by microbial metabolites such as SCFAs or secondary bile acids that influence the immune surveillance {Garcia-Castillo, 2016}. In liver, the inflammation consequent to the exposure, via the portal venous system, to intestinal bacterial components and metabolites can directly/indirectly lead to carcinogenesis {Yoshimoto, 2013; Ma, 2018}. In addition, the gut microbiota is thought to promote BCs through its role in steroid metabolism that increases the reabsorption of oestrogen, and its impact on energy metabolisms and obesity {Shapira, 2013}.

1.2.5.2 Gut microbiota and cancer therapy

The impact of gut microbiota on local and systemic antitumor immunity can also impact the outcome of anticancer therapy. So far, the influence of commensal bacteria on

the efficacy, as well as the toxicity, has been demonstrated for chemotherapy, immune checkpoint inhibitors, hematopoietic stem cell transplant (HSCT) and adoptive cell transfer (ACT), in preclinical and clinical studies (Table 1.2 and 1.3) {reviewed in McQuade 2019}.

Table 1.2 Preclinical evidence of the role of gut microbiota in anticancer therapies.

Treatment	Microbiota modification	Tumour model	Findings	Refs
Anti-PD-1 and/or anti-CTLA4	ABX cocktail	Fibrosarcoma (MCA-205), melanoma (RENCA), colon cancer (MC-38), Lewis lung carcinomas	Antibiotic exposure decreased anticancer immune responses after ICI and compromised survival	Routy, 2018a; Vétizou, 2015;
Anti-PD-L1	Different mice vendors	Melanoma (B16)	Microbiota composition influenced spontaneous antitumor immunity and anti-PD-L1 treatment efficacy	Sivan, 2015
CpG-ODN anti-IL-10R mAb or Oxaliplatin	ABX cocktail	Colon carcinoma (MC38), lymphoma (EL4)	Microbiota impacted systemic innate immune responses upon treatment with anti-IL-10R plus CpG-ODN therapy or oxaliplatin, affecting the response to therapy.	Iida, 2013
Cyclophosphamide	ABX cocktail	Fibrosarcoma (MCA-205)	Preventing translocation of <i>Enterococcus hirae</i> to secondary lymphoid organs decreased T cell stimulation and the efficacy of chemotherapy	Viaud, 2013; Daillere, 2016
Allo-HSCT	Single or combined antibiotics	GVHD after allo-HSCT in C57Bl/6 mice	Combination of piperacillin-tazobactam or imipenem after allo-HSCT associated with shorter survival durations	Shono, 2016
Gemcitabine	Ciprofloxacin	Colon carcinoma (MC26)	The gut microbiota contributed to chemotherapy resistance by altering drug metabolism	Geller, 2017
ACT from C57/Bl6 mice	Vancomycin, neomycin and metronidazole	TC1	Vancomycin stimulated CD8 α DC and sustained systemic adoptively transferred antitumor T cells. Treatment with neomycin and metronidazole had no effect on ACT.	Uribe-Herranz, 2018

ABX, antibiotic cocktail; ACT, adoptive T cell transfer; allo-HSCT, allogeneic haematopoietic stem cell transplantation; CpG-ODN, CpG oligodeoxyribonucleotide; CTLA-4, cytotoxic T lymphocyte protein 4; GVHD, graft-versus-host disease; ICI, immune-checkpoint inhibitors; IL-10R, IL-10 receptor; mAb, monoclonal antibody; MHC, major histocompatibility complex; PD-(L)1, programmed cell death protein (ligand)1; TC1, cervix and lung tumour cell line transformed by HPV E6/E7 viral oncogene {adapted from Routy, 2018b}.

Table 1.3 Clinical evidence of the role of gut microbiota in anticancer therapies.

Treatment	Patient cohort	Findings	Refs
Immunotherapy			
Anti-PD-1 or anti-PD-L1	249 patients with advanced-stage cancers (140 with NSCLC, 67 with RCC, and 42 with UC)	Antibiotic use associated with decreased response to ICI, <i>Akkermansia muciniphila</i> was found to be enriched in responders.	Routy, 2018a
Anti-PD-1	43 patients with metastatic melanoma	Better clinical outcome at 6 months and increased CD8+ T cells tumour infiltrate correlated with an enrichment of <i>Faecalibacterium</i> spp analysed in baseline faecal samples.	Gopalakrishnan, 2018
Anti-PD-1 or anti-CTLA4	42 patients with metastatic melanoma	Therapeutic benefit was correlated with a high abundance of <i>Bifidobacterium longum</i> , <i>Collinsella aerofaciens</i> , and <i>Enterococcus faecium</i> ; <i>Akkermansia muciniphila</i> was present exclusively in responders.	Matson, 2018
Anti-PD-1 (nivolumab)	74 patients with advanced-stage NSCLC	Prescription of antibiotics within 3 months before or during nivolumab treatment did not affect PFS.	Kaderbhai, 2017
Anti-CTLA-4 (ipilimumab) and/or anti-PD-1 (nivolumab)	Metastatic melanoma in two cohort (n=26 and n=39)	Baseline gut microbiota composition correlated with clinical outcome. Responders to ipilimumab plus nivolumab alone had faeces enriched for <i>Fecalibacterium prausnitzii</i> and <i>Dorea formicigenerans</i> , respectively.	Chaput, 2017
Anti-CTLA-4 (ipilimumab) and/or anti-PD-1 (nivolumab/ pembrolizumab)	39 melanoma patients	ICI responders were enriched in <i>Bacteroides caccae</i> and had faeces with a high content of anacardic acid	Frankel, 2017
Anti-PD-1/ anti-CTLA4 (nivolumab/ ipilimumab)	31 patients with metastatic RCC	The use of antibiotics before ICI treatment was a predictor of poor response	Ueda, 2019
Anti-PD(L)-1 and/or anti-CTLA4	196 cancer patients treated in routine clinical practice	Prior antibiotic therapy to ICI treatment was associated to a worse response and OS	Pinato, 2019
Anti-PD1 (nivolumab)	37 chinese patients advanced NSCLC enrolled in two clinical trials	The gut microbiota diversity correlated with the response to nivolumab. Responders exhibited enhanced memory T cell and NK cell signatures in peripheral blood cells	Jin, 2019
Chemotherapy			
Cyclophosphamide-based chemotherapy	800 patients with CLL and 122 patients with lymphoma	The use of antibiotics against Gram-positive bacteria during chemotherapy was associated with a reduced possibility to achieve objective response and with a shorter PFS.	Galloway-pena, 2016
Standard surgery and adjuvant chemotherapy	1069 CRC specimens	Microbiota composition and presence of <i>Fusobacterium nucleatum</i> , correlated with clinical outcome, independent of standard cancer staging.	Mima, 2016
Myeloablative and non-myeloablative chemotherapy regimens	500 patients with haematological malignancies undergoing allo-HSCT	Antibiotic prophylaxis altered microbiota diversity and correlated with increased incidence of acute GVHD and worse OS.	Routy, 2016

Myeloablative, reduced intensity and non-myeloablative chemotherapy regimens	Two cohort (n=541 and 113) with haematological malignancies undergoing allo-HSCT	Gut microbiota composition predicted clinical outcomes of allo-HSCT, <i>Eubacterium limosum</i> associated with decreased risk of relapse and progression, enrichment in <i>Blautia</i> spp. was associated with decreased GVHD-related death. Antibiotic treatment was associated with under-representation of <i>Blautia</i> spp.	Peled, 2017
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Allo-HSCT, allogeneic haematopoietic stem cell transplantation; AML, acute myeloid leukaemia; CLL, chronic lymphoblastic leukaemia; CRC, colorectal cancer; CTLA-4, cytotoxic T lymphocyte protein 4; FMT, faecal microbial transplantation; GVHD, graft-versus-host disease; ICI, immune-checkpoint inhibitors; NSCLC, non-small-cell lung cancer; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed cell death 1 ligand 1; PFS, progression-free survival; RCC, renal cell carcinoma; UC, Urothelial carcinoma {adapted from Routy, 2018b}.

Chemotherapy

Chemotherapeutics and immunotherapeutics drugs are affected by the gut microbiota composition which modulates the activity, efficacy and toxicity of several chemotherapy agents. In experimental models, the anticancer activity of cyclophosphamide is mediated by the induction of Gram-positive bacteria translocation into secondary lymphoid organs eliciting a Th17 and Th1 immune response {Viaud, 2013}. In a different study, the gut microbiota contributed to the efficacy and toxicity of oxaliplatin by modulating the release of proinflammatory cytokines in myeloid cells, a mechanism that was impaired in antibiotic-treated mice and germ free animals {Iida, 2013}. In addition, *Fusobacterium nucleatum*, beside promoting the development of colon carcinoma, it also mediates resistance to therapy by activating autophagy via TLR4 and MyD88 pathway {Yu, 2017}.

Besides the modulation of the antitumor immune response, the gut microbiota affects drug efficacy by regulating its metabolism. More than 40 drugs have been shown to be metabolized by the gut bacteria and, among them, the anticancer drugs methotrexate, gemcitabine and irinotecan are affected by the activity of bacterial enzymes {Spanogiannopoulos, 2016}. The efficacy of methotrexate, and antagonist of folic acid, is reduced by gut microbes due to its conversion from an active metabolite to a downstream metabolite with a reduced bioavailability {Haiser, 2013} by intestinal bacteria. Similarly, the efficacy of gemcitabine that is an antagonist of pyrimidines and acts by impairing the DNA synthesis, is negatively influenced by commensal bacteria. This drug functions within the

cell where it is activated and then transformed into the inactive metabolite difluoro-deoxy-uridine by cytidine deaminase enzyme and finally degraded. It was observed that *Gammaproteobacteria* in the intestinal lumen or in tumours (i.e. pancreatic tumours) produce a long isoform of this enzyme that may contribute to gemcitabine resistance by increasing its degradation {Galler, 2017}. In a different scenario, the toxicity of irinotecan, (CPT-11), a topoisomerase I inhibitor, is increased by gut microbiota {Wallace, 2010}. Irinotecan is a prodrug activated (SN-38 active form) by the cleavage of the side chain by enzyme carboxylase in plasma, intestinal lumen mucosae, liver and tumour cells. It is then inactivated by the uridine 5'-diphospho-glucuronosyltransferase (SN-38G inactive form) and excreted into the intestinal lumen with bile acids. However, during the transit through the intestinal lumen, the SN-38G isoform can be converted back into the active form by the enteric bacteria β -glucuronidase enzymes consequently increasing the intestinal toxicity of this drug {Wallace, 2010}. The modulation of gut microbiota is starting to be considered a new strategy to alleviate drug toxicity in patients, in the case of irinotecan the specific inhibition of bacterial β -glucuronidase reduced severe diarrhoea in mice {Wallace 2010; Guthrie, 2017}. Furthermore, 5-fluorouracil-induced mucositis was ameliorated by using probiotics bacterial strain {Yeung, 2019} supporting the possibility to use the gut microbiota to fight chemotherapy-induced side effects.

Immunotherapy and immune checkpoint blockade

Preclinical studies showed the involvement of the gut microbiota in the response to immune checkpoint inhibitors such as anti-PD-(L)1 and anti-CTLA-4 mAbs and how modulation of intestinal ecology enhanced the therapeutic response {Vétizou, 2015; Sivan, 2015}. The association between response to immunotherapy and commensal bacterial have also been described in patients across cancer type {McQuade, 2019}. Antibiotics assumption represented a negative prognostic factor for patients undergoing immune checkpoint inhibitors treatment {Routy, 2018a; Derosa, 2018} and also lower microbial

diversity or altered composition were associated to impaired response to therapy {Routy, 2018a; Gopalakrishnan, 2018; Matson, 2018; Chaput, 2017; Frankel, 2017}. In the case of anti-CTLA4 therapy, the baseline differences in the gut microbiota composition associated, in multiple cohorts, with the possibility to develop immune-related adverse event, in particular colitis {Chaput, 2017; Dubin, 2016}.

Other anticancer treatments

The gut microbiota has a role in cancer therapies other than chemotherapy and immunotherapy. ACT is a form of immunotherapy whereby activated immune cells (T cell or NK cells) are transferred into the host to directly attack the tumour. The modulation of the gut microbiota by vancomycin has been shown to sustain transferred T cells in an IL-12 dependent manner {Uribe-Herranz, 2018}. The broad spectrum antibiotics that are used in the setting of HSCT can induce a pronounced dysbiosis that impairs the gut integrity: the loss of beneficial bacteria (e.g. *Faecalibacterium*, *Ruminococcus*, *Lactobacillus*, and *Blautia*) have been associated with severe graft versus host disease and transplant related mortality in patients {Andermann, 2016; Mathewson, 2016}.

Similar to other cancer therapies that induce immunogenic cell death, radiotherapy causing cell death promotes systemic inflammation. The gut microbiota has been demonstrated to have a role in both response to therapy {Colbert, 2017} and radiotherapy-induced toxicity {Crawford, 2005}.

Overall, two different aspects of the gut microbiota contribution to the therapeutic efficacy of anticancer treatments have emerged: on one hand, treatment-induced damages of the intestinal epithelial barrier allow translocation of bacteria from intestinal lumen to secondary lymphoid organs exerting an adjuvant effect on immune cells sustaining their activation against cancer. On the other hand, local intestinal microbiota provides tonic signals that prime immune cells creating conditions for an effective response to therapy {Gorjifard, 2016} (Fig. 1.13).

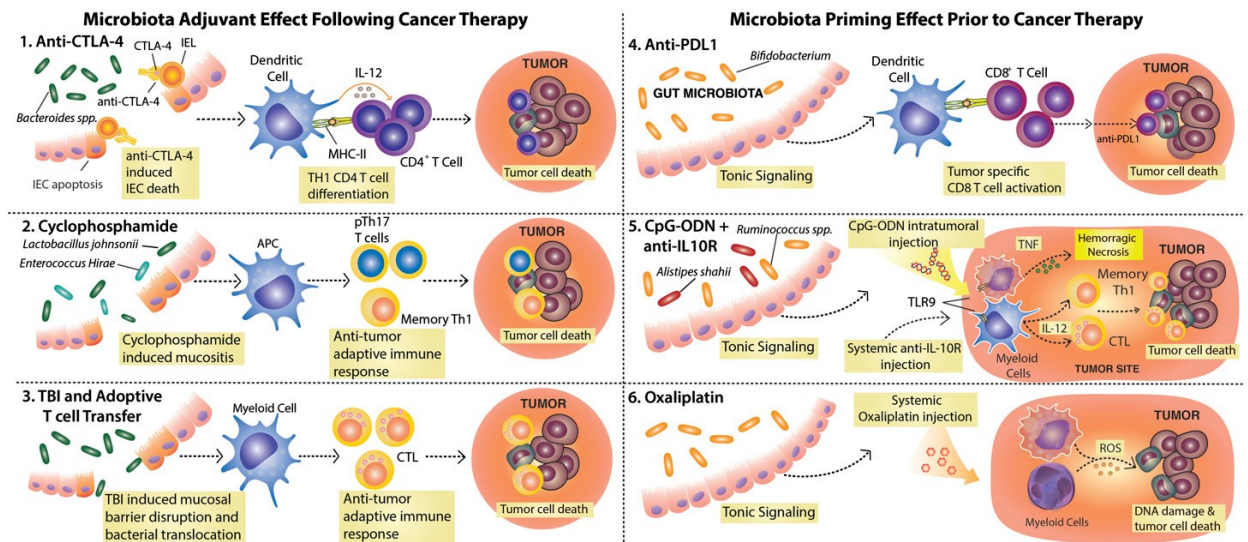


Figure 1.13 Impact of commensal bacteria on anticancer therapies. (1-3) Adjuvant effect of the gut microbiota in treatments that cause damages in the intestinal barrier allowing for bacteria translocation and stimulation of myeloid cells. (4-6) Priming effect of the gut microbiota to cancer therapy, tonic signals from the intestinal ecosystem shape host immunity and impact the response to therapy {Gorjifard, 2016}.

1.2.5.3 Gut microbiota: biomarker or new treatment strategy?

Microbiota as biomarker

Studies in preclinical models and human cohorts showed that the diversity and the composition of gut microbiota are associated with therapeutic efficacy in different forms of cancer (Table 1.1 and 1.2). This evidence sustains the potential use of gut microbiota as response biomarker to be considered alongside other biomarkers already in use. To date the potential use of gut microbiota signature has been described by Dr. Wargo's group {Gopalakrishnan, 2018}: in this study, high basal abundance of *Clostridiales* and low abundance of *Bacteroidales* characterized responsive melanoma patients under anti-PD-1 treatment. The profiling of gut, tumours, and other microbiota is now being integrated into the design of clinical trials for several type of cancer therapies but standardization of sample collection, methods used to sequence the samples (16S rRNA sequencing, metagenomics, metatranscriptomics and metabolomics profiling), and analysis pipelines represent relevant source of variability among institutions and common guidelines will be required to advance the biomarkers research. Generally, the composition of microbiota will

be considered as a complementary prognostic or predictive biomarker to be integrated with other known correlates of clinical outcome.

Microbiota as therapeutic intervention

The evidence from preclinical models that modulation of intestinal microbiota can enhance therapeutic response to immunotherapy (i.e. *Bifidobacterium* {Sivan, 2015} *Akkermansia muciniphila* {Routy, 2018a}) and chemotherapy (i.e. *Enterococcus hirae* and *Barnesiella intestinihominis* {Daillère, 2016}), open the possibility to exploit the manipulation of gut microbiota to improve the efficacy of cancer therapies {McQuade, 2019}. Intervention strategies to manipulate intestinal microbiota of cancer patients can range from less precise to a more specific approaches (Fig. 1.14).

The faecal microbiota transplantation (FMT) consist in the transfer of a large community of bacteria and is already used for the treatment of resistant *Clostridium difficile* infectious, and it is under clinical evaluation in cancer patients {reviewed in Routy, 2018b}. FMT can be performed orally or rectally by using lyophilized and encapsulated faeces from donor without cancer (or responsive to the treatment) to patients. The difficulty to find the optimal donors (including the lack of definition of favourable microbiota), to collect sufficient material to enable long-term and prolonged treatment, alongside the possibility to transfer disease-promoting bacteria such as obesity/carcinogenesis related bacteria, make the FMT a challenging strategy to modulate intestinal microbiota.

The existing commensal community, can be shaped via prebiotics or dietary changes to favour the expansion of beneficial bacteria. Prebiotics are defined as “a substrate that is selectively utilized by host microorganisms conferring a documented health benefit” {Gibson, 2017}. An example of prebiotic is represented by the fructooligosaccharides while an unresolved question is about whether non-digestible fibre and all non-starch polysaccharides should be considered as prebiotics or not {Hutkins, 2016}. Nonetheless,

this definition of prebiotics expands the concept of prebiotics to possibly include non-carbohydrate substances such as polyphenols and polyunsaturated fatty acids, and their direct application to body sites other than the gut (i.e. vaginal tract and skin) {Gibson, 2017}.

A modification of the gut microbiota is also possible through the administration of probiotics, “live organisms that, when administered in adequate amounts, confer a health benefit on the host” {Hill, 2014}, they are generally produced by the fermentation of food and most of them come from two groups, *Lactobacillus* and *Bifidobacterium*, although many other types of bacteria are classified as probiotics (e.g. *Enterococcus faecium*, *Enterococcus durans*, *Streptococcus thermophilus* and *Bacillus subtilis*) {Floch, 2014}. The combination of prebiotics with probiotics to create “synbiotic” mixture is possible for an optimal expansion of favourable bacteria {Klaenhammer, 2012}.

In theory, antibiotics could be also considered to reduce the abundance of harmful bacteria in the gut but they lack the precision to modulate very specific bacterial populations. In a more precise approach, single or defined mixture of cultivable bacterial species can be used as adjuvant strategy in anticancer therapies, but attention should be paid to create stable ecosystem of cooperating species avoiding biological resources or ecological niches completion among the selected bacteria {Foster, 2017}. If a bacterial metabolic pathway is identified together with the metabolic products favourable to improve antitumor immunity and immunotherapy, small molecules derived from bacteria can be suitable as immune-modulatory drugs (Fig. 1.14).

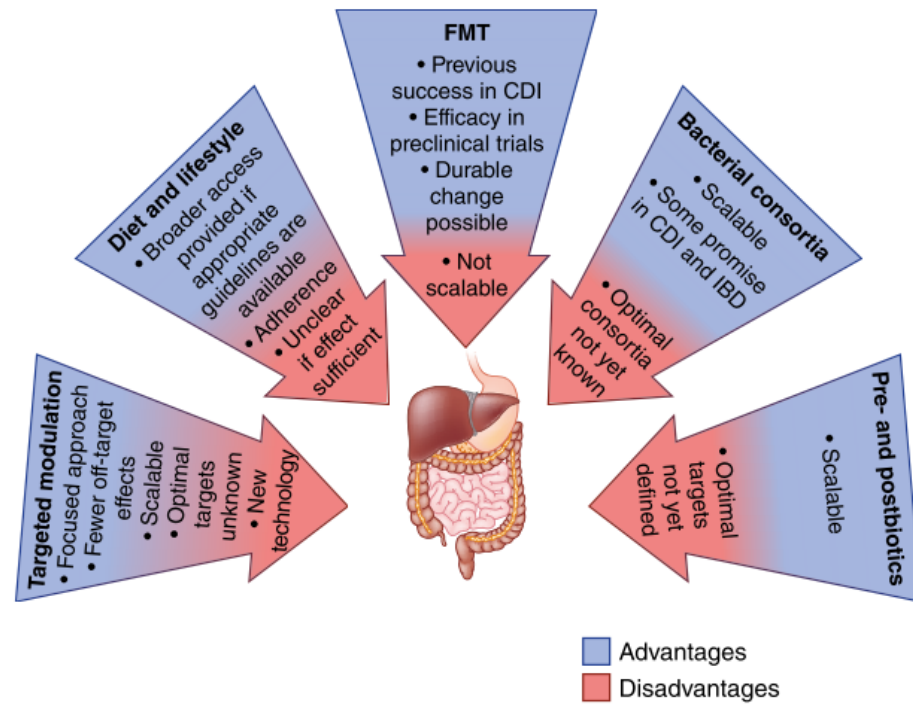


Figure 1.14 Strategies to target gut microbiota. To improve the response to treatment in cancer patients different strategies can be adopted to modulate the intestinal microbiota. For each strategies pro (blue) and cons (red) are illustrated {Helmink, 2019}.

Chapter 2: SCOPE OF THE THESIS

The introduction of the anti-HER2 targeted therapy considerably advanced the treatment of women with HER2-positive BC. Despite the increment in survival benefit, a large number of patients progress upon the treatment even within the group of tumours prone to get advantage from trastuzumab treatment (HER2-enriched by PAM50 classifier and highly infiltrated by immune cells) rising the clinical need to understand the reason underlying this resistance. Moreover, existing biomarkers, such as the expression levels of ERBB2 and ESR1 genes, the intrinsic subtypes, and tumour immune infiltration or immune signatures, are able to predict the response to trastuzumab in only half of the patients. Suggesting the possibility of tumour extrinsic factors playing a role in the efficacy of anti-HER2 treatment.

The gut microbiota has been recently investigated as a mediator of anticancer treatment, so far clinical and preclinical evidence showed that different intestinal microbiota status is associated with the efficacy of immunotherapy and certain chemotherapies that elicit an immune response in their mechanism of action. This evidence open the possibility to use the gut microbiota as both intervention strategy to improve the anticancer treatment efficacy or as a predictive biomarkers.

As regard to trastuzumab, this mAbs, in addition to inhibit its target HER2, exerts a cytotoxic activity by recruiting the immune cells within the tumour microenvironment. Considering the relevance of immune cells in trastuzumab cytotoxicity and the role of commensal bacteria in the development and maintenance of the immune system, a possible role for the gut microbiota was hypothesize as a tumour extrinsic factors that contributes to the heterogeneity observed in trastuzumab efficacy. The ultimate goal of this project was to investigate the role of gut microbiota in the treatment of HER2-positive BC and the specific aims of my work were:

- Study the influence of commensal bacteria on trastuzumab efficacy in preclinical models of HER2-positive BC;
- Investigate the possibility to exploit microorganism(s) to confer sensitivity to trastuzumab treatment;
- Study the role of the gut microbiota composition in HER2-positive patients treated with neoadjuvant trastuzumab.

Chapter 3: MATERIALS AND METHODS

3.1 Patients cohort

Patients with stage II-III HER2-positive breast cancer that received neoadjuvant trastuzumab-based therapy between 2017-2019 at the Istituti Clinici Scientifici Maugeri Spa-SB, Pavia, Italy, were enrolled in this study in collaboration with Dr. Fabio Corsi. Women received four cycles of Adriamycin/cyclophosphamide followed by four to six cycles of paclitaxel or docetaxel/trastuzumab (AC-TH, where A, Adriamycin; C, cyclophosphamide; T, taxanes and H, trastuzumab). Two patients belonging to this cohort were treated with only TH starting from the beginning. The pCR was defined as no residual invasive tumour in the breast at the time of the surgery. To study the association between gut microbiota composition and trastuzumab treatment, faecal samples were collected before trastuzumab administration. To preserve the viability of anaerobic bacteria, faeces were collected using the ANAEROGENTM compact SYSTEM (Oxoid Microbiology Products, Thermo Fischer Scientific, Waltham, MA, USA). For metagenomics analysis, 1 g aliquots of faeces were immediately frozen at -80°C .

For the gene expression analysis on formalin-fixed and paraffin-embedded (FFPE) specimens, the leftover material of samples collected during standard surgical and medical procedures was used. Samples were donated by the patients to the Institutional Biobank for research purpose, and aliquots were designated for this study after approval by the institutional review board and a specific request to the independent committee of the institutes Istituti Clinici Scientifici Maugeri (Pavia) and Fondazione IRCCS Istituto Nazionale dei Tumori (Milan) (Observational study n°22/16).

Based on the relevance of the use of antibiotics in determining the gut microbiota content, patients were asked to report changes in the usual habits and antibiotics assumption.

3.2 Antibiotics treatment and *in vivo* experiments

3.2.1 Cell lines and reagents

The murine MI6 tumour cell line {Castagnoli, 2014} was established from spontaneous primary mammary carcinomas developed in a transgenic human $\Delta 16$ HER2-LUC FVB female mice ($\Delta 16$ HER2), a model that express the constitutively active splice variant $\Delta 16$ of the human HER2 proto-oncogene under the MMTV promoter {Marchini, 2011}. MI6 cells were maintained in complete culture medium (MammoCult; Stemcell Technologies, Vancouver, Canada) supplemented with 1% foetal bovine serum (FBS; Sigma-Aldrich, MO, USA), penicillin 1 $\mu\text{g/mL}$ and streptomycin 1 $\mu\text{g/mL}$ (Sigma-Aldrich), hydrocortisone 0.48 $\mu\text{g/mL}$ (Stemcell Technologies), heparin 2 U/mL (Sigma-Aldrich) and cultured at 37°C in a 5% CO₂ atmosphere incubator. The cell line D2F2/E2 derived from the transfection with the human HER2 gene under the CMV promoter of the D2F2 cell line {Piechoki, 2001}, established from a spontaneous mammary tumour that arose in a BALB/c. D2F2/E2 cells were cultured in DMEM (GIBCO, Thermo Fisher Scientific, Massachussets, USA) supplemented with 10% FBS, 1% sodium pyruvate and nonessential amino acids under constant selection with G418 800 $\mu\text{g/mL}$ (Geneticin, Thermo Fisher Scientific). The TUBO cell line derived from spontaneous lobular mammary tumour developed in BALB/c mice transgenic for the transforming rat *neu* gene {Rovero, 2000}. TUBO were cultured in RPMI (GIBCO) supplemented with 10% FBS.

3.2.2 Antibiotics treatment

To study the impact of intestinal microbiota alteration on the efficacy of anti-HER2 inhibition, vancomycin hydrochloride (vancomycin; Cayman Chemical, MI, USA) and streptomycin sulphate salt (streptomycin; Sigma-Aldrich) were dissolved in the drinking water at a concentration of 200 mg/L. For the experiments performed at the National Institute of Health (NIH), an antibiotic cocktail (ABX) was used to completely disrupt the intestinal microbiota of BALB/c mice, antibiotics were dissolved in the drinking water supplemented with 0.01% sweetener (sucralose Splenda ®): vancomycin 500 mg/L,

neomycin sulphate (neomycin; Sigma-Aldrich) 700 mg/L, Primaxin (Imipenem and Cilastin, Merck&Co., NJ, USA) 500 mg/L or ampicillin sodium salt (ampicillin, Sigma-Aldrich) 1 g/L. ABX were administered to mice *ad libitum* starting from age four-weeks and changed every 48-72 h with fresh solution.

3.2.3 *In vivo* experiments

Mice were maintained in laminar-flow rooms at constant temperature and humidity, with food and water given *ad libitum*. Experimental protocols used for animal studies were approved by the Ethics Committee for Animal Experimentation (OPBA) of Fondazione IRCCS Istituto Nazionale dei Tumori of Milan and by the Italian Ministry of Health in accordance with institutional guidelines (projects no. 11/16 and 11/17). Experiments conducted at the National Institute of Health (NIH) – National Cancer Institute (NCI) of Bethesda, MD, US, were approved by the NCI-Bethesda Animal Care and Use Committee (protocol no. CIP-004-O/P).

Female FVB mice or BALB/c mice (four-weeks old; body weight, 16-18 g) were purchased from Charles River Laboratories (Calco, Italy). Mice were administered an antibiotic (vancomycin or streptomycin or ABX, prepared as described in the antibiotic treatment paragraph) or sterile water, as control. After four weeks of antibiotic treatment, 1×10^6 MI6 cells were injected into the mammary fat pad of FVB mice. The anti-HER2 mAb trastuzumab [Herceptin] (Genentech, Roche, Basel, Switzerland) was administered via intraperitoneal injection (i.p.) at a concentration of 5 mg/kg body weight, starting when tumours reached a palpable volume (~3 mm of diameter). Trastuzumab antitumor efficacy was evaluated as inhibition of tumour growth. Similarly, 5×10^5 TUBO or D2F2/E2 cells were injected into the mammary fat pad of BALB/c mice. Animals harbouring TUBO tumours received 100 µg of 7.16.4 mAb (InVivo mAb, BioXcell, Lebanon, NH, USA) twice a week for two weeks. Mice bearing D2F2/E2 tumours were treated twice a week with trastuzumab (5 mg/kg body weight). Untreated mice received 100 µL of NaCl solution

(0.9%) as control. Tumours were measured once a week, and the volume was calculated as $0.5 \times d1^2 \times d2$, where d1 and d2 are the smaller and larger diameters, respectively.

A breeding colony of Δ16HER2 mice was maintained in the Animal Facility of Fondazione IRCCS Istituto Nazionale dei Tumori. Ear biopsies were harvested from four-weeks old mice for the routine genotyping. DNA was extracted using the Exgene Tissue DNA mini kit (GeneAll® kit, Tribioscience, California, USA) and analysed by PCR (primers: F, 5'-GGCTCAGTGACCTGTTTTGG-3' and R, 5'-TGATGAGGATCCCAAAGACC-3'), with an expected amplicon length of 231 bp. Positive female mice were divided into two groups and one was administered vancomycin (200 mg/L) in the drinking water and one was kept as control. Mice were inspected weekly by palpation to monitor tumours onset and growth. Mice were treated with i.p. injection of trastuzumab at a concentration of 8 mg/kg body weight {Castagnoli, 2014} or NaCl solution (0.9%) starting from age 8 weeks or after tumour onset (~3 mm of diameter) according to the experiments. The antitumor efficacy of trastuzumab was assessed by evaluating tumour onset, tumour multiplicity and volume.

3.2.4 Faecal microbiota transplantation

The causal link between trastuzumab efficacy and gut microbiota composition was evaluated with a FMT experiment. The intestinal microbiota of recipient FVB mice was depleted by treating animals for four weeks with an antibiotic cocktail (ABX) containing vancomycin (500 mg/L), neomycin, ampicillin and metronidazole (Cayman Chemical) (1 g/L) starting at four-weeks of age. Due to the reluctance of BALB/c mice to drink the antibiotic solution, the depletion of their gut microbiota was performed by daily oral gavage of 200 µL ABX solution (vancomycin 5 mg/mL, neomycin, metronidazole and ampicillin 10 mg/mL) for ten consecutive days {Adachi, 2017}. For the FMT, fresh faeces (130-150 mg) were resuspended in 1 mL of pre-reduced PBS 1X in order to preserve the viability of the anaerobic bacteria. Faeces were then homogenized by vortexing the sample

in presence of a 3 mm diameter tungsten carbide bead (QIAGEN, Hilden, Germany) and centrifuged at 100 rpm for 1 min to avoid big particles. Mice were transplanted using 200 μ L of faecal suspension twice a week for two weeks and then once a week until the end of the experiment.

The total bacterial load and the viability of faecal samples used for the FMT were evaluated by flow cytometry. The concentration of cells was determined by using the Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA): the cell suspensions were double-stained with SYBR green I 1x (Sigma-Aldrich) and propidium iodide (PI) 0.5 μ g/mL (Sigma-Aldrich), at 37°C for 15 min in the dark, in order to distinguish alive, injured and dead cells based on their fluorescence. The SYBR-Green I and the PI fluorescence of stained cells was recovered in the FL-1 channel (excitation 488 nm, emission filter 530/30 nm) and FL-3 Channel (excitation 488 nm, emission filter 630/30 nm), respectively.

For the FMT experiment using faecal material from BC patients, 2 g of faeces collected using the ANAEROGENTM compact SYSTEM (Oxoid) were frozen after being homogenized in 10 mL of autoclaved brain-heart-infusion media (BHI, Sigma) supplemented with 0.1% L-cysteine (Sigma) and 15% glycerol. Faecal suspension was kept for 1 hour at room temperature inside the ANAEROGENTM compact SYSTEM bag (Oxoid) to remove oxygen and, then stored in 1 mL aliquots. The FMT in mice was performed using 200 μ L of thawed faecal suspension (7-8 mice for each patient). The FMT was performed for a total of five times, 24-48 h before and after tumour cells injection, and then every ten days for additional three times. Trastuzumab treatment started when tumour reached a palpable volume and the mAbs was administered twice a week for 3 weeks at a concentration of 5 mg/kg.

3.2.5 Lactic acid producing bacteria treatment

Lactococcus lactis subsp *IL1403* (*L. lactis*) and *Lactobacillus paracasei* FMB (*L. paracasei*) were provided by Dr. Stefania Arioli from the Dept. of Food, Environmental and Nutritional Sciences (DeFENS) at the Università degli Studi of Milan. Bacteria were cultured anaerobically, *L. lactis* was grown in the M17 broth (Difco Laboratories INC., Detroit, MI, USA) supplemented with 1 % (w/v) glucose (Sigma-Aldrich) (gM17) at 30°C and *L. paracasei* was grown in the Man-Ragosa-Sharpe (MRS) broth (Difco) at 37° C.

LAB suspensions to be used in *in vivo* treatment were prepared starting from an overnight culture, LAB were then washed twice with sterile saline solution (0.9% NaCl), and resuspended, at the concentration of 5×10^9 cells/mL determined by using an Accuri C6 flow cytometer, in saline solution in presence of 10% (v/v) glycerol to preserve cells viability during storage at -80°C. Mice were transplanted using 200 µL of thawed suspension three times a week throughout the entire experiment.

3.3 Cellular biology

3.3.1 Immunofluorescence analyses

Immunofluorescence (IF) analyses were performed to detect trastuzumab distribution within the tumours. Tumour samples were embedded in the O.C.T. compound (AgarScientific Ltd, Stansted, UK) and stored at -80°C. Tissue specimens were cut on the cryostat microtome and sections were fixed with 4% paraformaldehyde for 20 min at room temperature. Tumours sections were then incubated with a blocking solution with 3% BSA before being stained with the anti-human AlexaFluor®568 secondary antibody at a dilution of 1:100 for 1 hour at room temperature. Nuclei were counterstained with DAPI (Thermo Fischer Scientific). Images were acquired at the microscopy facility of Fondazione IRCCS Istituto Nazionale dei Tumori using a confocal laser scanning microscope Leica TCS SP8 X (Leica Microsystems GmbH, Mannheim, Germany). The fluorochrome was excited by a pulsed super continuum White Light Laser (470-670nm; 1nm tuning step size). In

particular, AlexaFluor-568 was excited selecting 557 nm laser line and detected from 562 to 642 nm; and DAPI excited with 405 nm diode laser and detected from 422 nm to 488 nm. Four images were acquired for each sample in the scan format 512x512 using HC PL APO 40X/1.3 CS2 oil immersion objective and a pinhole set to 1 Airy unit. Data were analysed using the public available ImageJ software for image processing.

3.3.2 Immunohistochemistry analyses

The expression of HER2 was evaluated by immunohistochemistry (IHC) on FFPE tumour tissue from no antibiotic and vancomycin or streptomycin treated mice collected at the end of the experiment. Tumour slides were deparaffinised and serially rehydrated, the antigen retrieval was performed using 10 mM citrate buffer, pH 6 at 96°C for 6 min. Slides were then stained with the rabbit anti-p185 (clone A0485 - Dako, Agilent Technologies, Santa Clara, CA, USA) at 1:150 dilution. Immunoreactions were visualised using streptavidin-biotin-peroxidase (Thermo Fisher Scientific) and the 3,3' Diaminobenzidine (DAB) Chromogen System followed by counterstaining with haematoxylin. Quantification was performed using the public available ImageJ software for image processing.

The characterization of the tumour immune infiltrate was carry out by Dr. Tripodo at the Dept. of Human Pathology of the Università degli Studi of Palermo. For the IHC analyses four-micrometre-thick FFPE tissue section were deparaffinised and rehydrated. The antigen unmasking was performed using Novocastra Epitope Retrieval Solutions pH 9 (Novocastra, Newcastle Upon Tyne, UK) in a PT Link pre-treatment module (Dako, Denmark) at 98°C for 30 min. The sections were brought to room temperature and washed by a specific protein block (Novocastra) the slides were incubated over night with the primary antibodies at 4 C°. Table 3.1 lists all the molecules evaluated and the antibodies used for the staining. The staining was revealed by AEC (3-amino-9-ethylcarbazole) substrate-chromogen following producer's instruction. The slides were counterstained with Harris haematoxylin (Novocastra). All the sections were analysed under an AXIO Scope

A1 optical microscope (ZEISS, Oberkochen, Germany) and 5 sections were counted at 40x magnification microphotographs were collected through an Axiocam 503 Colour digital camera (Zeiss) using the Zen2 software.

Table 3.1 List of antibodies used for IHC analyses

Molecule	Host	Clone	Dilution	Brand
CD45	Rabbit	ab10558	1:1000	Abcam
CD3	Rabbit	ab5690	1:100	Abcam
CD4	Rat	4SM95	1:100	eBioscience
CD8	Rat	4SM15	1:100	eBioscience
GranzymeB	Rabbit	760-4283	Ready to use	Vector
ArginaseI	Rabbit	GTX109242	1:200	Genetex
CD31	Mouse	1A10	1:50	Leica Novocastra
Gr-1	Rat	RB6-8C5	1:100	BioLegend

3.3.3 Flow cytometry analyses

Tumours were harvested at the end of the experiment for the immune phenotype analysis. Samples were cut into small pieces and digested in DMEM medium containing Collagenase IV (100 µg/mL) and DNase I (200 U/mL) for 50 min at 37°C under constant agitation. The tumour suspension was filtered through a cell strainer with 40 µm pore diameter. The red blood cells lysis was performed by incubating tumour cells suspension with ACK (Sigma) on ice for 5 min. Tumour pellet was then resuspended in 1 mL of FACS buffer (PBS 1x, 2% FBS and 2mM EDTA), 200µL of cells suspensions were stained with primary antibody directly conjugate with fluorophores (Table 3.2 and 3.3) for 15 min at 4°C. Dead cells were excluded using the LIVE/DEAD Fixable Blue (or Near-IR) Dead Cell Stain Kit (Thermo Fisher Scientific). Stained samples were fixed 15 min at 4°C with 1% formalin and run on a BD LRS Fortessa SORP II (BD Bioscience) cytometer at the flow cytometry facility of the National Institute of Health (NIH) (Bethesda, MD, USA)

or on a BD FACSCanto system (BD Bioscience) at the flow cytometry facility of the Fondazione IRCCS Istituto Nazionale dei Tumori. The analysis was then performed using the FlowJo software (Tree Star, Ashland, OR, CA, USA).

Table 3.2 Antibodies for flow cytometry BD LRS Fortessa SORP II

Molecule	Conjugate	Clone	Dilution	Brand
CD45	BUV737	104	1:100	BD Biosciences
CD45	eFluor450	30-F11	1:100	eBioscience
CD3	PerCPCy5.5	17A2	1:200	Biolegend
CD4	BUV395	GK1.5	1:100	BD Biosciences
CD8	APC Cy7	53-6.7	1:100	BD Biosciences
TCR β	APC	H57-597	1:200	BD Biosciences
CD19	BV650	1D3	1:200	BD Biosciences
CD49b	PE	DX5	1:200	Biolegend
CD44	BUV737	IM7	1:100	BD Biosciences
CD62L	BV786	MEL-14	1:200	BD Biosciences
MHCII	BV605	2G9	1:1000	BD Biosciences
TCR $\gamma\delta$	FITC	GL3	1:100	BD Biosciences
CD24	BV650	M1/69	1:100	BD Biosciences
Ly6G	PECy5	1A8	1:200	BD Biosciences
Ly6G	BUV395	1A8	1:100	BD Biosciences
Ly6C	Alexa Fluor647	HK1.4	1:1000	Biolegend
CD86	BV786	GL1	1:200	BD Biosciences
F4/80	BV711	T45-2342	1:300	BD Biosciences
F4/80	PECy5	BM8	1:200	BD Biosciences
CD11c	PerCp Cy5.5	N418	1:200	Biolegend

CD11b	PE-CF594	M1/70	1:500	BD Biosciences
CD206	PECy7	MMR	1:200	Biolegend
CD64	PE	X54-5/7.1	1:200	BD Biosciences

Table 3.3 Antibodies for flow cytometry BD FACSCanto

Molecule	Conjugate	Clone	Dilution	Brand
CD3	FITC	145-2C	1:10	MACS
CD49b	PE	DX5	1:100	MACS
CD4	PEVio770	GK.15	1:10	MACS
CD8	PECy5	53.6.7	1:100	eBioscience
CD19	APC	6D5	1:10	MACS
CD11b	PE	ICRF44	1:100	BD pharmingen
CD11c	PECy7	N418	1:100	BD pharmingen
F4/80	PerCPCy5.5	BM8	1:100	eBioscience
Ly6C	APC	1G7.G10	1:10	MACS
Ly6G	FITC	1A8	1:10	MACS

3.4 Metagenomic analyses and molecular biology

For murine studies, faeces were collected from 10 mice per group of treatment before the beginning of trastuzumab administration or at the end of the experiment and were conserved at -80°C. The metagenomic DNA was extracted from one stool pellet. For the analysis of gut microbiota in patients, the DNA was extracted from 200 mg of faeces conserved at -80°C.

In the analysis of faecal microbiota sample, several aspects should be taken into consideration as possible sources of variation. Faecal sample preparation and storage can introduce bias during downstream analyses affecting the 16S rRNA gene sequencing. Our study was conducted on faecal samples conserved at -80°C for short/long period (<2

years) according to the experiment. Although freezing sample has been shown to cause an increment in the relative abundance of *Bacteroidetes* and *Firmicutes* as compared to freshly analysed samples {Bahl, 2016}, the most critical factor that influences the results on bacteria composition remains the method used for the DNA extraction {Videnska, 2019}. In addition, samples stored for long term (>2 years) at -80°C have demonstrated enough stability to be considered still largely representative of the original community {Shaw, 2016; Tap, 2019}. For these reasons, we do not expect relevant biases derived from storage conditions or extraction methods in our analyses of 16S rRNA gene profile. In fact, the same DNA extraction kit was used, and all samples were stored at -80°C for a period of time lower than 2 years thus inter-individual variation should be much greater than that attributable to storage conditions.

3.4.1 Microbial DNA extraction

The metagenomic DNA was extracted by using the DNeasy PowerLyzer PowerSoil DNA isolation kit (QIAGEN, Hilden Germania) according to the following adapted protocol: samples were transferred to the *PowerLyzer glass bead tubes* and 750 µL of *Bead solution* were added and the samples vortexed. Then 60 µL of *Solution C1* were added, vortexed and heated 10 min at 65° C. Feaces were homogenized using the TissueLyzer II machine (QIAGEN) for 5 min with oscillation frequency of 30/sec. Samples were then centrifuged at 10000 rpm for 10 min at room temperature. *Solution C2* and *C3* were added simultaneously and incubated at 4° C for 5 min. Samples were then centrifuged and 1200 µL of *Solution C4* were added to the supernatant and vortexed. Samples were passed through the *Spin Filters* by centrifugation at 10000 rpm. *Solution C5* (500 µL) was added to wash the filters and DNA was eluted in 50 µL of DNase and RNase - free H₂O. The DNA extracted from faecal samples was quantified using a Qubit 2.0 fluorimeter (Invitrogen, Thermo Fisher Scientific).

3.4.2 16S rRNA gene sequencing and analyses

To analyse the composition of the bacterial community the 16S rRNA gene was profiled. The DNA extracted was normalized to a final concentration of 5 ng/ μ L and a fragment encompassing the variable regions 3 and 4 (V3 and V4) of the 16S rRNA gene was amplified from 12.5 ng of total DNA and sequenced on the MiSeq Illumina Technology (Illumina, Inc, San Diego, CA, USA) platform at the Centre for Life Nanosciences, Italian Institute of Technology (Rome, Italy). The raw data of the DNA sequencing were analysed in collaboration with Dr. Simone Guglielmetti and Dr. Giorgio Gargari at the DeFENS (Università degli Studi of Milan), using the open-source bioinformatics Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 {Caporaso, 2010}. Bacterial abundances in each faecal sample were reported at the taxonomic level of genus. The ecological diversity (alpha and beta) were calculated by QIIME software version 1.9.1. The α -diversity of microbial composition was analysed using three different indexes Chao1, Shannon and Simpson indexes: Chao1 index weighs the richness of different bacteria present in each sample; Shannon and Simpson indexes are mainly influenced by the evenness plus richness and to the evenness respectively.

Principal component analyses (PCoA) were done based on UniFrac distance matrices {Lozupone, 2005} to visually compare the microbiota of the different treatment groups considering bacterial phylogenetic distances. In the analysis of patient's β -diversity, the unsupervised clustering by JSD algorithm was performed: the Calinski-Harabasz index (CH) together with Silhouette method of interpretation and validation of consistency within clusters data was applied to figure out the best clustering possible represented with the ellipse in the β -diversity.

Linear discriminant analysis (LDA) effect size analysis (LEfSe) was performed to identify differentially abundant taxa {Segata, 2011} in groups of treatment of R and NR patients. Specifically, the algorithm uses the non-parametric factorial Kruskal-Wallis sum-

rank test associated with a p-value correction test to detect features with significant differential abundance with respect to the group of interest. Statistical significance was set at $p \leq 0.05$, and mean differences with $0.05 < p \leq 0.10$ were accepted as trends.

3.4.3 RNA extraction and qRT-PCR

To evaluate the expression of intratumor cytokines, RNA was extracted from frozen tumours. Samples were transferred in vials containing 1 mL of QIAzol Lysis Reagent (QIAGEN) and homogenized with the TissueLyser system (QIAGEN) with the help of a 3 mm tungsten carbide bead (QIAGEN) inside the vials. Samples were centrifuged at 1500 rpm at 4°C and the supernatant transferred to new vials. RNA was extracted according to manufacturer's instructions. The cDNAs were reverse transcribed from 2 µg of total RNA by using the High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific), the gene expression level was assessed by qRT-PCR using the TaqMan probe-based assays and the TaqMan Fast Universal PCR Master Mix (Applied Biosystem, Thermo Fisher Scientific) on the ABI Prism 7900HT sequence detection system (Applied Biosystem, Thermo Fisher Scientific). The total 16S rRNA in faecal samples was quantified by using the SYBR Green PCR Master Mix (Applied Biosystem, Thermo Fisher Scientific). Table 3.4 lists the TaqMan Gene Expression Assays and probes that were used.

Table 3.4 List of Taqman assays and probes used for qRT-PCR analysis

Gene Taqman assay	Code
<i>Ifng</i>	Mm01168134_m1
<i>Il12a</i>	Mm00434169_m1
<i>Il15</i>	Mm00434210_m1
<i>Il6</i>	Mm00446190_m1
<i>Il10</i>	Mm01288366_m1
<i>Tgfb1</i>	Mm01178820_m1
<i>Gapdh</i>	Mm99999915_g1

Probe	Sequence
<i>U16S-Forward</i>	5'-ACTCCTACGGGAGGCAGCAGT-3'
<i>U16S-Reverse</i>	5'-ATTACCGCGGCTGCTGGC-3'

3.4.4 Gene expression profile

GEP on patients tumour biopsies were performed in collaboration with the Functional Genomic and Bioinformatic Facility of our Institute. RNA was extracted from FFPE samples using miRNAeasy FFPE kit (QIAGEN). After quality check and quantification by 4200 TapeStation (Agilent Technology) and Qubit Fluorometer (Thermo Fisher Scientific), respectively, RNA expression was assessed using the human Affymetrix Clariom S Pico assay (Thermo Fisher Scientific). The single-stranded cDNA samples for hybridization was generated by 100 ng of total RNA. Then, cDNA was enzymatically fragmented and biotinylated using the WT Terminal Labeling kit (Thermo Fisher Scientific), combined with the hybridization buffer, and injected into the Human Clariom S arrays targeting >20,000 well-annotated genes. Arrays were stained using the Affymetrix® GeneChip® Fluidics Station 450 and scanned with the 7G Affymetrix® GeneChip® Scanner 3000. Raw data were processed using Transcriptome Analysis Console software (Thermo Fisher Scientific). CEL files containing feature intensity values were converted into summarized expression values by Robust Multi-array Average (RMA) which consists of background adjustment, quantile normalization across all chips. All samples passed QC thresholds for hybridization, labelling and the expression of housekeeping gene controls. The data will be deposited into the Gene Expression Omnibus repository. The research-based PAM50 subtypes predictor was applied using the publicly available algorithm as described after merging of the dataset with 50 consecutive BC cases profiled on the same platform in our Institute, performing median centring of the PAM50 genes {Prat, 2014a}.

3.5 Microbial metabolic analyses

Short-chain fatty acid (SCFAs) and lactate were measured in faecal and plasma sample. The quantification of faecal SCFAs in mice under vancomycin and streptomycin treatment, was performed in collaboration with Dr. Claudio Gardana at the Università degli Studi of Milan. Approximately 100 mg of faecal sample was suspended in 2 mL of a solution 0.001% HCOOH in water containing 2-MBA (IS, 50 μ molar). After vortexing for 1 min, the suspension was centrifuged at 10,000 x g for 1 min, and the supernatant was collected into a 5 mL flask. The residue was treated with 2 mL of a solution 0.001% HCOOH in water containing 2-MBA (50 μ molar), vortexed for 1 min and centrifuged at 10,000 x g for 1 min. The supernatant was pooled; the volume was adjusted to 5 mL with 0.001% HCOOH in water containing 2-MBA (50 μ molar) and the solution stored at -20°C until analysis. Determination of SCFAs concentration was achieved through ultraperformance liquid chromatography-high resolution-mass spectrometry analysis (UPLC-HR-MS). UPLC-HR-MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with an Exactive Orbitrap MS with an HESI-II probe for electrospray ionization (Thermo Fisher Scientific) {Gargari, 2016}.

The analysis of SCFAs from murine plasma samples and faecal sample of HER2-positive BC patients were performed by the company ProDigest (Gent, Belgium).

Lactate quantification was performed using commercially available enzymatic assay kit (Lactate Assay kit, r-biopharm, Boehringer Mannheim, Germany).

3.6 Statistical analyses

Analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc, CA, USA). Differences between groups were determined using the two-tailed unpaired *t* test. Differences among α -diversity in microbial samples were determined by the Mann-Whitney-Wilcoxon test. Statistical significance was set at $p < 0.05$, and mean differences with $0.05 < p \leq 0.10$ were accepted as trends.

Chapter 4: RESULTS

4.1 Impact of gut microbiota on trastuzumab efficacy in the FVB murine model

4.1.1 Trastuzumab efficacy in antibiotic-treated FVB mice

The role of commensal bacteria in the therapeutic benefit of anti-HER2 treatment, was investigated in antibiotic-treated FVB mice, that harboured MI6 cell tumours, a HER2-positive cell lines established from spontaneous mammary tumours developed in $\Delta 16\text{HER2}$ FVB female mice {Castagnoli, 2014}. Vancomycin and streptomycin were used in this study based on their poor intestinal absorption {Rao, 2011; Krause, 2016}. Vancomycin is mainly directed against Gram-positive bacteria acting as inhibitor of the peptidoglycan biosynthesis {Hammes, 1974}, while streptomycin is a broad spectrum antibiotics that inhibits the proteins synthesis in bacteria causing cell death {Luzzato, 1968}. The administration of antibiotic in mice started at age four-weeks and continued for the entire duration of the experiment, and MI6 cells were injected into the mammary fat pad of mice after four weeks of treatment. The therapy with trastuzumab started when tumours reached a palpable volume, and its efficacy was evaluated measuring tumour growth inhibition (Fig. 4.1). Antibiotic abrogated trastuzumab efficacy as compared to control (no antibiotic, NoA) animals.

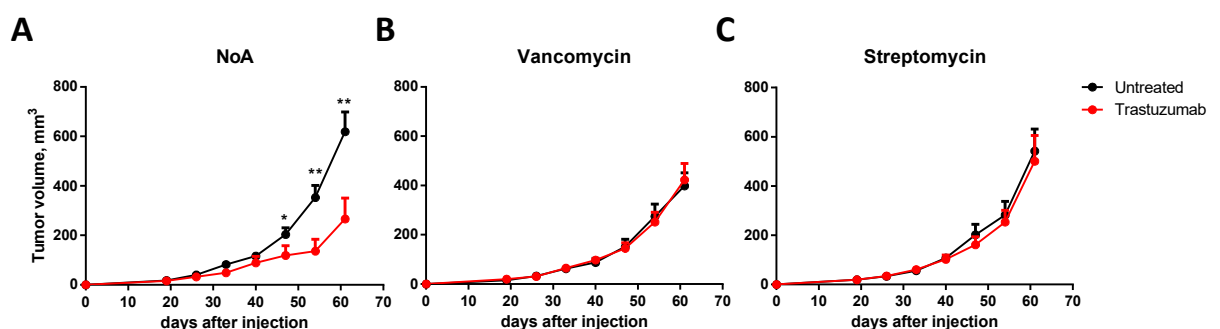


Figure 4.1 Efficacy of trastuzumab in antibiotic-treated mice. A-C) Tumour growth measurement in control mice (NoA) (A), vancomycin (B), or streptomycin (C) (200 mg/L) treated mice. Trastuzumab was administered i.p. at a concentration of 5mg/kg body weight twice a week starting when tumours reached a palpable volume. (Black, tumor growth in untreated mice; red, tumour growth in mice treated with trastuzumab). * $p < 0.05$ and ** $p < 0.01$ by unpaired Student's t-test.

To assess whether antibiotics have an impact on tumours and could possibly impair the binding of trastuzumab to its target, HER2 expression, tumour vasculature and the mAb distribution within the tumour were evaluated (Fig. 4.2 and 4.3). IHC analyses carried out of FFPE tumour specimens (Fig. 4.2 A), did not reveal differences in HER2 expression among the three groups of treatment (Fig. 4.2 B). Furthermore, since abnormalities in the tumour vasculature can have negative consequences on the homogeneous drugs distribution within the tumour {Klein, 2019} and certain classes of antibiotic are reported to have anti-angiogenic properties {Miyake, 2019}, we evaluated whether vancomycin and streptomycin had an impact on tumour vessels by staining specimens for CD31, a transmembrane glycoprotein expressed on endothelial cells (Fig. 4.2 A). The count of CD31+ cells, showed that the oral treatment with vancomycin and streptomycin did not alter the number of tumour vessels (Fig. 4.2 B).

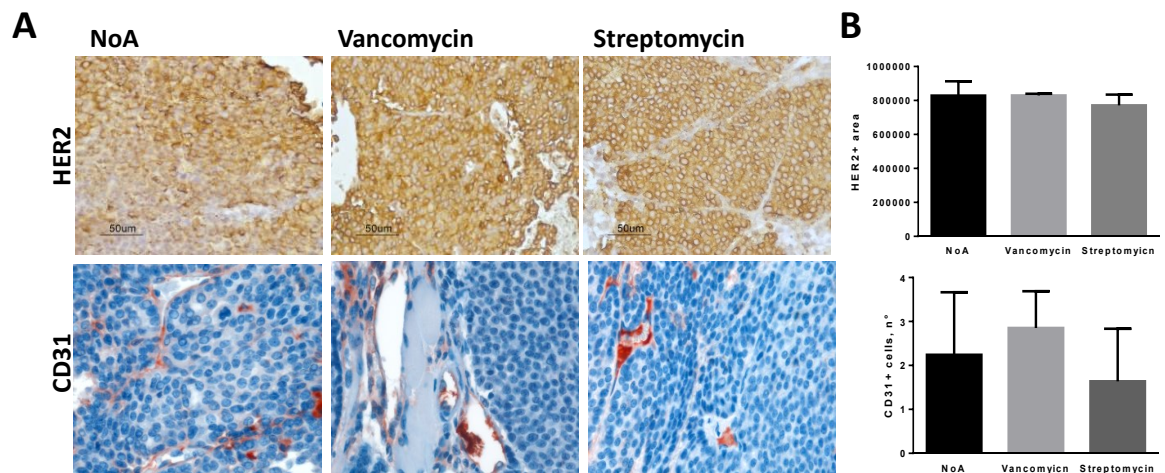


Figure 4.2 Characterization of tumour microenvironment in antibiotic-treated mice. A) Evaluation of HER2 expression and CD31+ cells by IHC. B) Quantification of HER2 by using the ImageJ software, and count of CD31-positive cells.

Furthermore, IF analysis on OCT-frozen tumours samples revealed no differences in trastuzumab distribution within the tumours of vancomycin-treated mice as compared to controls (Fig. 4.3 A-B).

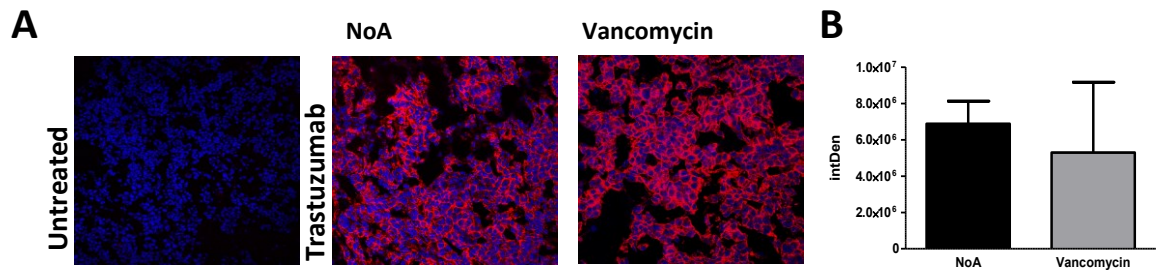


Figure 4.3 Trastuzumab distribution in tumours. **A)** Representative images of trastuzumab distribution in tumours from control (NoA) and vancomycin treated-mice as evaluated by IF using an antibody specific for the human IgG1. Blue: dapi, red: trastuzumab. **B)** Quantification of red pixels by using Image J software on 4 images per group (data shown as mean \pm SD).

Altogether these data ruled out a possible antibiotic-induced tumour changes that led to the inefficient interaction between trastuzumab and its target.

4.1.2 Analysis of antibiotic-induced alterations of the intestinal microbiota

4.1.2.1 Metagenomic analysis of gut microbiota composition

The structure of the intestinal bacterial community was evaluated in mice under vancomycin or streptomycin treatment. Stool samples were collected before the beginning of the treatment with trastuzumab and the gut microbiota composition was analysed by sequencing the 16S rRNA gene on the Illumina MiSeq platform. The treatment with antibiotic significantly reduced taxonomic richness, as reflected by the lower α -diversity indexes Chao1, Shannon and Simpson (Fig. 4.4 A) in vancomycin and streptomycin-treated animals compared to controls. Vancomycin had the strongest impact on α - and β -diversity, although also streptomycin induced relevant changes in the gut microbial composition as shown by the PCoA of the β -diversity (Fig. 4.4 B and C).

A detailed phylogenetic analysis revealed that bacterial taxa belonging to the *Actinobacteria* and *Firmicutes* phyla decreased upon antibiotic administration. In addition, vancomycin treatment induced a substantial loss of members from the taxonomic phyla *Bacteroidetes*, while the relative abundance of phyla such as *Proteobacteria* and *Verrucomicrobia* was found to be increased. Inside the phylum *Firmicutes*, both antibiotics

determined the reduction of numerous taxa belonging to the order of *Clostridiales*, and particularly to the family *Lachnospiraceae*. (Fig. 4.4 D).

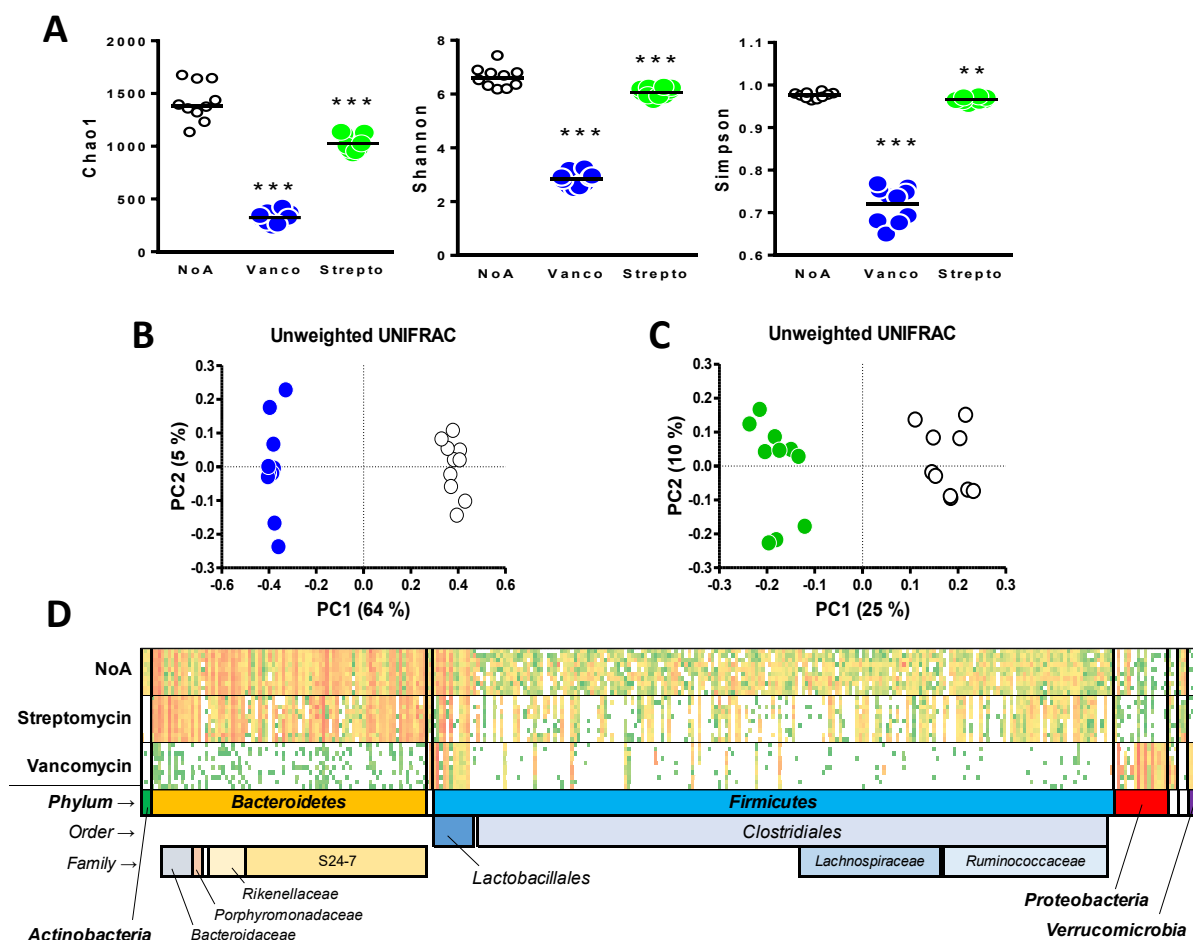


Figure 4.4 Antibiotic-induced changes in the intestinal microbiota. Analysis of gut microbiota composition by sequencing the 16S rRNA gene in faeces from control (NoA), vancomycin or streptomycin-treated mice. **A)** Microbial α -diversity between control and vancomycin or streptomycin-treated mice calculated by Chao1, Shannon and Simpson indexes, **p<0.01 and ***p< 0.001 by Mann-Whitney-Wilcoxon test. **B-C)** Principal coordinate analysis plot of microbial β -diversity between control (white) group and vancomycin (**B**, blue) or streptomycin (**C**, green) treated animals generated using unweighted UniFrac algorithm. **D)** Heatmap of bacterial taxa relative abundance in the three groups of treatment. Green (low)-red (high) relative abundance, white, not detected.

To further explore these findings, a high-dimensional class comparison using linear discriminant analysis effect size (LEfSe) was performed in order to identify differences in the bacteria abundance among the three groups (Fig. 4.5 A and B). *Firmicutes* taxonomic phylum, in particular families from the *Clostridiales* taxonomic order (e.g. *Ruminococcaceae* and *Lachnospiraceae*), *Bacteroidales* (e.g. *Rikenellaceae* and

Prevotellaceae) were represented only in control animals (red) while the guts of vancomycin (blue) and streptomycin (green) treated mice were mainly colonized by members of *Proteobacteria* plus *Verrucomicrobia*, and *Bacteroidetes*, respectively.

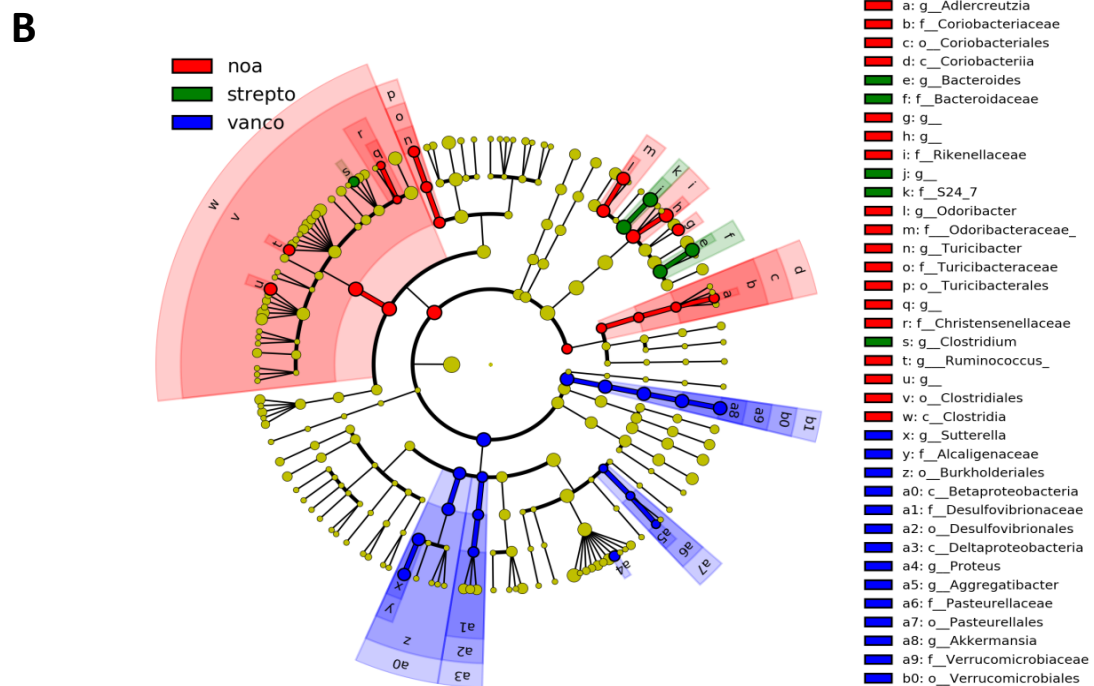
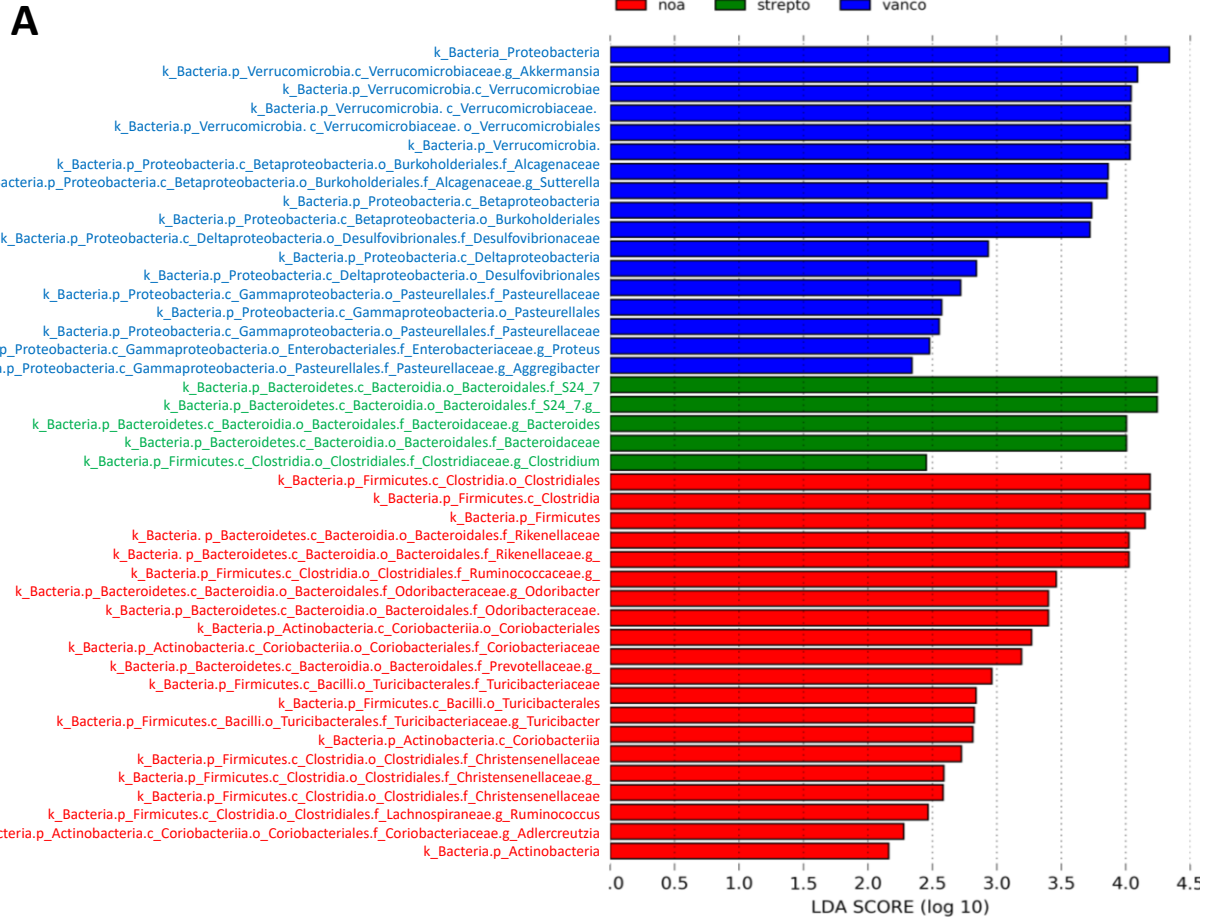


Figure 4.5 Compositional differences in the gut microbiota of antibiotic-treated mice. Differences in the gut microbiota of vancomycin or streptomycin treated FVB mice were analysed by high-dimensional class comparison using linear discriminant analysis effect size (LEfSe). The significant differences among group of treatment were highlighted. Control (NoA, red), streptomycin (green) and vancomycin (blue). **A)** Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the faecal microbiomes, length indicates effect size associated with a taxa. **B)** Taxonomic cladogram from LEfSe showing differences in faecal bacteria, the background colour indicates which bacterial order is differentially abundant in each group of treatment. Dot size is proportional to the bacteria abundance.

4.1.2.2 Quantification of bacterial metabolites in faecal and blood samples

Based on the key role of bacterial metabolites and SCFAs in the communication between gut microorganisms and host, the impact of vancomycin and streptomycin on SCFAs production was analysed in faeces and blood samples of treated mice. Consistently with the strong modification of the intestinal microbiota composition, the levels of butyrate, propionate, and acetate were markedly decrease in vancomycin-treated animals (Fig. 4.6 A), while a high variability was observed in samples from streptomycin group. Furthermore, in the attempt to link the modification occurred in the gut to the systemic impact of intestinal microbiota modification, butyrate, propionate, and acetate were quantified in the plasma of antibiotic treated mice (Fig. 4.6 B). The analysis revealed a decrease in the amount of circulating SCFAs in animals under antibiotic treatment, however, the interpretation of these results is limited by the low amount of material used for the analysis (200 μ L) that gave result values considered below the limit of quantification (LOQ).

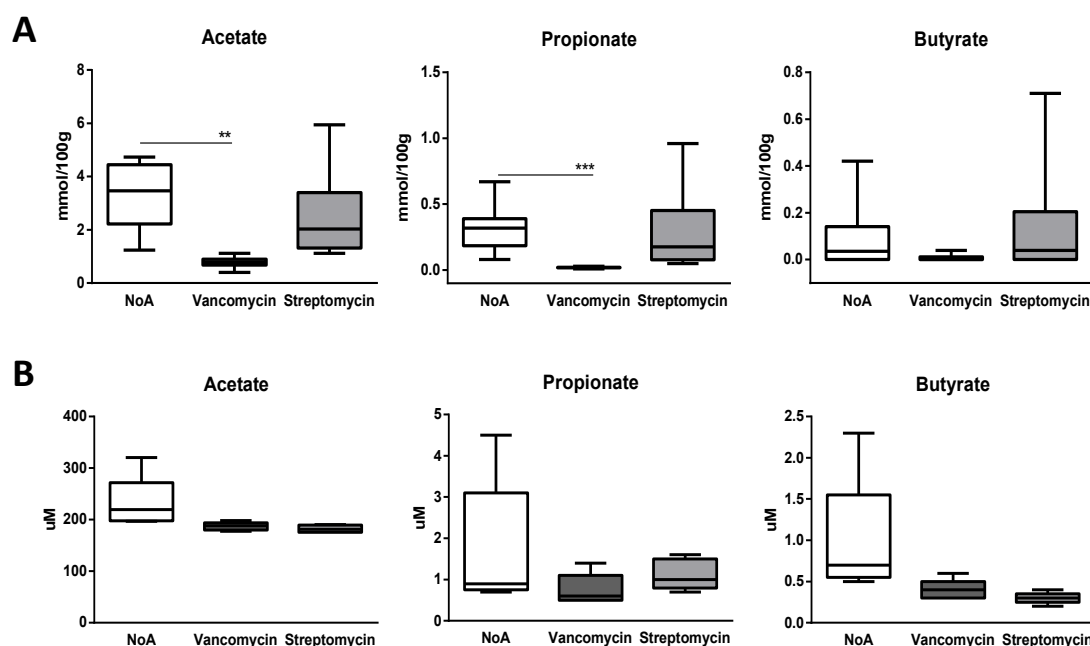


Figure 4.6 Short chain fatty acids quantification in antibiotic-treated mice. Products of bacteria metabolism were analysed in faecal (A) and plasma (B) samples. The analysis was performed by ultraperformance liquid chromatography- high-resolution-mass spectrometry analysis (UPLC-HR-MS). Results obtained for plasma samples were <LOQ .** p<0.01; *** p<0.001 by unpaired Student's t-test.

Together with SCFAs, the amount of lactic acid was quantified in faeces of antibiotic treated mice. In our model antibiotic-induced modification of intestinal microbiota resulted in a strong decrease in the levels of total lactate (Fig. 4.7).

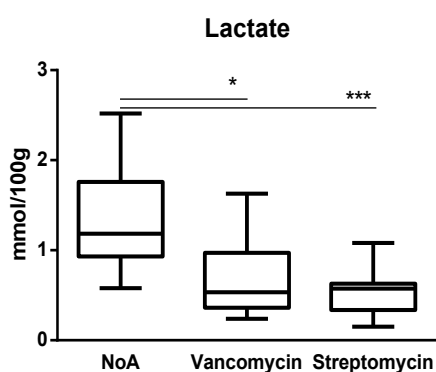


Figure 4.7 Lactic acid quantification in antibiotic-treated mice. Lactate was analysed in faecal sample from vancomycin or streptomycin-treated mice by using the lactate-quantification kit. * p<0.05; *** p<0.001 by unpaired Student's t-test.

The data obtained demonstrated alterations in intestinal metabolites following antibiotic treatment and, even though these results should be considered with caution, they indicate that perturbation of the intestinal microbiota had systemic consequences that may explain how gut microbiota modification impact the efficacy of the anti-HER2 treatment in

a distant tumour site. Furthermore, changes occurred in the levels of lactic acid let us hypothesise the possibility to improve trastuzumab efficacy by using lactate producing bacteria (as shown in section 4.1.6).

4.1.3 Characterization of the tumour immune microenvironment in FVB mice

4.1.3.1 Immunohistochemistry analysis of the tumour immune infiltrate

Tumours of antibiotic-treated mice were characterized by IHC for immune cell infiltration. Since the efficacy of trastuzumab has been described to be mediated by cells of the innate and adaptive immunity, tumours were stained for CD45, CD3, CD4, CD8 granzyme B (GZMB), Gr-1 (granulocytic marker) and Arginase I (M2-like maker) in collaboration with Dr. Tripodo at the Dept. of Human Pathology of the Università degli Studi of Palermo. The count of stained cells was reported as a total number or as a count of intratumor or stromal cells (Fig. 4.8).

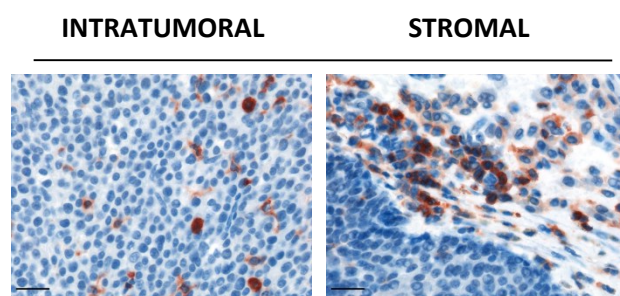


Figure 4.8 Immune cells localization within the tumour analysed by IHC. Representative IHC staining of intratumor and stromal CD45+ immune infiltrate. Original magnification 40x. Scale bar 50 μ m.

The staining showed a common increment in the total number of CD45+ immune cells in tumours of antibiotic-treated mice (Fig. 4.9 A). The administration of vancomycin and streptomycin was associated with a lower number of tumour infiltrating CD3+ lymphocytes (Fig. 4.9 B) while the myeloid tumour infiltrate as analysed by the Gr-1 staining, was found to be increased (Fig. 4.9 C).

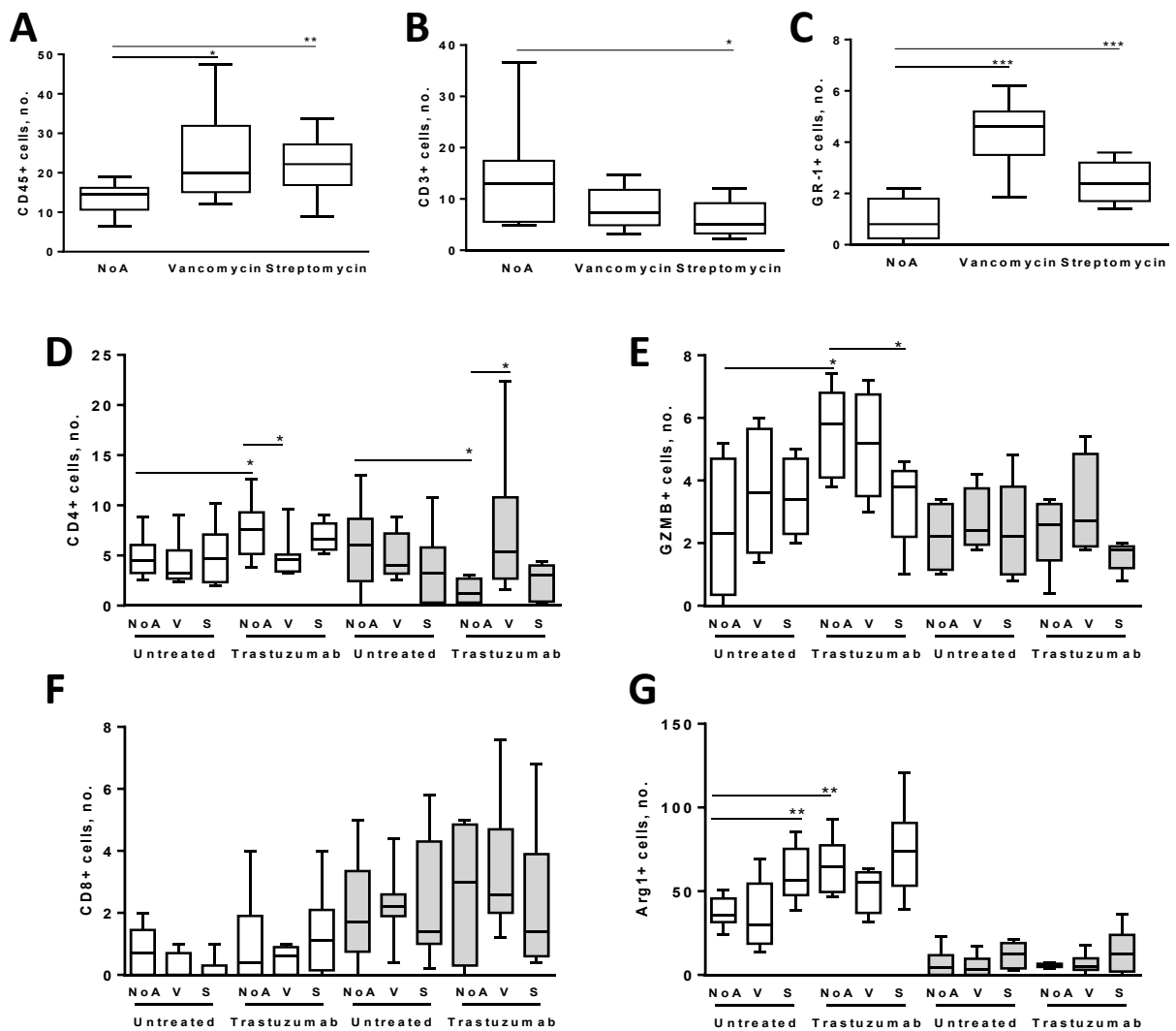


Figure 4.9 Analysis of tumour immune infiltrate in antibiotic treated mice. Tumour samples were characterized by IHC for the immune cell infiltrates. A-C) Count of total CD45+ (A), CD3+ cells (B), Gr-1+ cells (C). D-E) Intratumor and stromal staining of CD4+ T cells (D), and granzyme B (GZMB) positive (E). Control (NoA), vancomycin (V) or streptomycin (S) treated mice treated or not with trastuzumab. Intratumor (white) and stromal (grey). (Data are shown as box and whiskers plot, min to max) * $p<0.05$; ** $p<0.01$ and *** $p<0.001$ by unpaired Student's t-test.

Interestingly, trastuzumab increased the number of intratumoral CD4+ T cells associated with their concomitant decrease in the stromal compartment (Fig. 4.9 D). The redistribution of CD4+ cells in the tumour microenvironment was observed only in the group of control animals but not in mice under vancomycin or streptomycin regimen. Similarly, the number of GZMB+ cells increased upon the anti-HER2 treatment in the tumours of control mice, but not in those under antibiotic regimens (Fig. 4.9 E). The IHC

staining also revealed a low number of CD8⁺ T cells that infiltrate MI6 tumours, and mostly localized in tumour stroma (Fig. 4.9 F) and differently from the other immune subset no intratumoral increment of the CD8⁺ T cells number was observed upon trastuzumab administration even in control mice. Arginase I is usually analysed as marker of M2-like macrophages but can also be expressed by neutrophils, the basal number of Arg1⁺ cells was increased in tumours from streptomycin treated mice as compared to tumours of control mice, suggesting the establishment of an immunosuppressive microenvironment. We also observed the increment of Arg1⁺ cell upon trastuzumab in control mice, this may reflect an accumulation of macrophages/neutrophils in the tumour microenvironment after several treatments with the mAb, as the tumours were collected at the end of the experiment.

4.1.3.2 Analysis of intratumor cytokines expression

The expression of pro- and anti-inflammatory cytokines was analysed by quantitative PCR in tumours to add information to the immune characterization performed by IHC. Trastuzumab treatment promoted the expression of proinflammatory cytokines in control animals: (Fig. 4.10): a trend towards the increment of *Ifng* (p=0.08) and a concomitant increase of *Il12a* and *Il15* expression were found. IL12a and IL15 are two cytokines relevant for trastuzumab activity and they are released by macrophages and mononuclear cells, respectively, to induce proliferation and activation of NK cells and Th1 lymphocytes. Furthermore, no modulation of *Il12a* and *Il15* was seen upon trastuzumab treatment in antibiotic-treated mice despite their higher basal expression levels as compared to tumours of control animals (Fig. 4.10 A-C). No modulation of *Il6* and *Il10* can be appreciated in this analysis while tumours of vancomycin-treated mice presented higher expression of *Tgfb* than controls (Fig. 4.10 D-F).

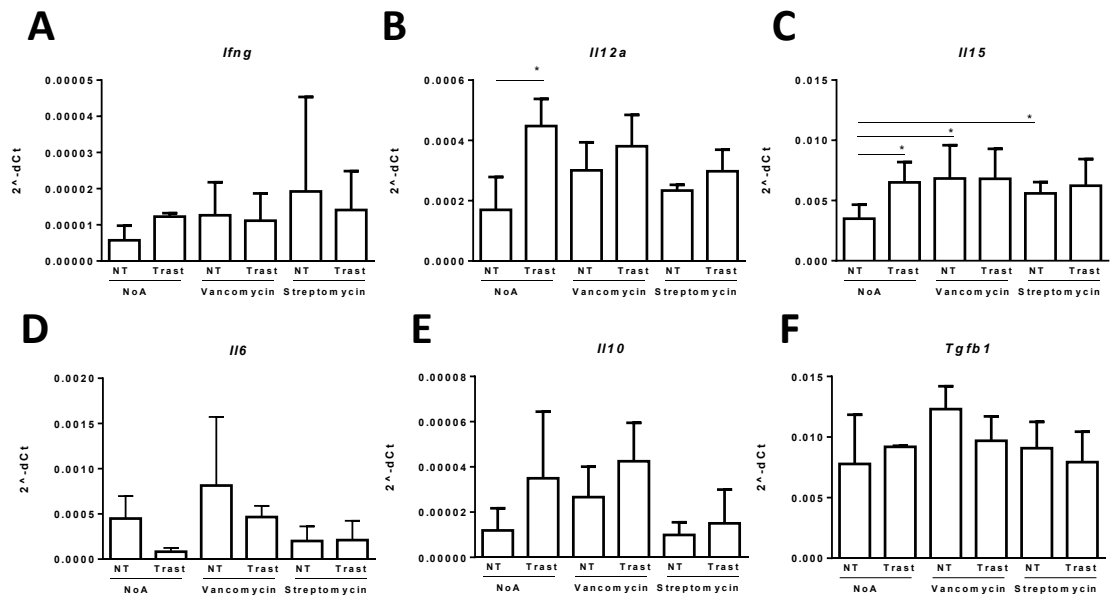


Figure 4.10 Evaluation of intratumoral cytokines expression. RNA was extracted from frozen tumours and the expression of *Ifng* (A) , *Il12a* (B), *Il15*(C), *Il6* (D), *Il10* (E), and *Tgfb1* (F) was analysed by qRT-PCR. * $p < 0.05$ by unpaired Student's t-test.

Overall, the characterization of tumour immune microenvironment revealed an association between changes in the gut microbiota composition and tumour immune infiltrate. Further analysis focused on immune cells activation and *ad hoc* experiment should be performed in future to address the mechanism underlying the influence of commensal bacteria on tumour immune microenvironment.

4.1.4 Causal role of the gut microbiota in trastuzumab efficacy

4.1.4.1 Faecal microbiota transplantation

To explore the cause-effect relationship between gut microbiota composition and trastuzumab anti-tumour activity, a FMT experiment was performed. The intestinal microbiota of FVB mice was depleted with a cocktail of broad-spectrum antibiotics (ABX) (vancomycin 500 mg/L, neomycin, ampicillin and metronidazole 1 g/L) before being transplanted with faecal suspension from donor antibiotic-treated or control mice. The efficiency of intestinal microbiota depletion was assessed after four weeks of ABX treatment by flow cytometry and analysis of the 16S rRNA gene (Fig. 4.11). Faeces were homogenized and stained for the quantification by flow cytometry: an average of 2×10^9

event/mL versus 3.4×10^6 event/mL were observed respectively in control versus ABX faecal suspensions (Fig. 4.11 A). In parallel, the quantification of the total 16S rRNA gene by qRT-PCR confirmed a significant decrease in the total bacterial load in ABX-treated mice (Fig. 4.11 B). The efficiency of FMT was assessed by flow cytometry by analysing the viability of bacteria contained in faecal suspension and faeces collected from recipient mice, before and 24h after the faecal transplant (Fig. 4.11 C). As shown in figure, bacteria harvested from donor mice maintained viability and were efficiently transferred to recipient animals.

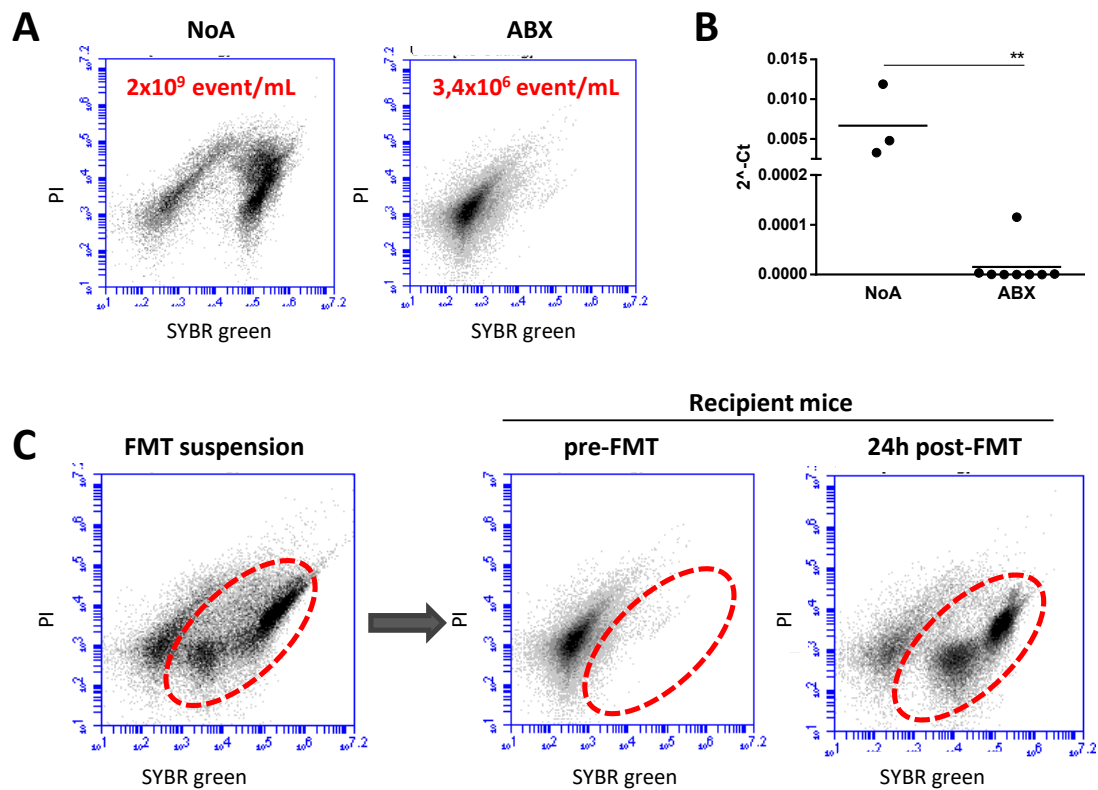


Figure 4.11 Quantification of total bacterial load in ABX-treated mice. A cocktail of broad-spectrum antibiotics (ABX) (metronidazole, ampicillin, neomycin 1 g/L, vancomycin 0.5 g/L) was administered in the drinking water of four-weeks old FVB female mice for a period of 28 days to deplete their intestinal microbiota. **A)** Equal amount of faeces from control (NoA) and ABX-treated mice were homogenized in pre-reduced PBS (130 mg/mL) and it was stained with propidium iodide (PI) and SYBR green and quantified by flow cytometry. The average count of three replicates is shown **B)** Bacterial load quantification by qRT-PCR using universal primers for 16S rRNA gene. ** $p < 0.01$ by unpaired Student's t-test. **C)** Representative images of donor stool suspensions and faecal samples from recipient mice as analysed by flow cytometry before and after FMT to assess bacteria viability and transplant efficiency. Red: transferred bacteria population.

The therapeutic efficacy of trastuzumab was then assessed in FMT-mice bearing MI6 tumours and we observed that the inhibition of tumour growth upon anti-HER2 treatment was more effective in mice transplanted with faeces from control animals (FMT-NoA) than in FMT-vancomycin mice (Fig. 4.12 A-B) while in FMT-streptomycin mice only an initial unresponsiveness to the treatment was observed (Fig. 4.12 C).

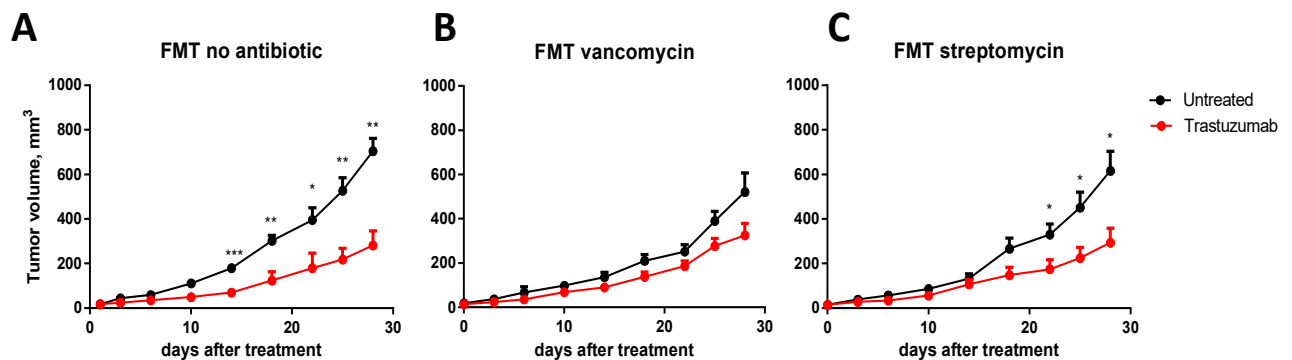


Figure 4.12 Trastuzumab efficacy in gut microbiota transplanted mice. The intestinal microbiota of four-weeks old FVB mice was depleted by using ABX administered in the drinking water (vancomycin 0.5 g/L, metronidazole, ampicillin and neomycin 1 g/L) for four weeks. Then, MI6 cells were injected into the mammary fat pad of ABX-treated mice and their microbiota was reconstituted via FMT using faecal suspension from donor mice treated or not with vancomycin or streptomycin. FMT was performed twice a week for two weeks and then once a week for the entire duration of the experiment. Trastuzumab was administered biweekly (at a concentration of 5 mg/kg body weight) starting when tumour reached a palpable volume. Untreated (black) and trastuzumab (red). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired Student's t-test.

4.1.4.2 Analysis of β -diversity in FMT-mice

The intestinal ecology of FMT-mice was evaluated by sequencing the 16S rRNA gene in faeces collected at the end of the experiment for each group of treatment. The analysis of β -diversity was performed based on UniFrac distant matrix and the PCoA plot showed that, samples from FMT-vancomycin mice segregated more distant from control mice than samples from FMT-streptomycin mice (Fig. 4.13). Suggesting that the robust modifications of intestinal microbiota induced by vancomycin were better recapitulated in FMT-mice (Fig. 4.13 A) and maintained until the end of the experiment than in FMT-streptomycin mice (Fig. 4.13 B). The similarity between FMT-NoA and FMT-

streptomycin can possibly explain why FMT-streptomycin mice responded to trastuzumab at the end of the experiment.

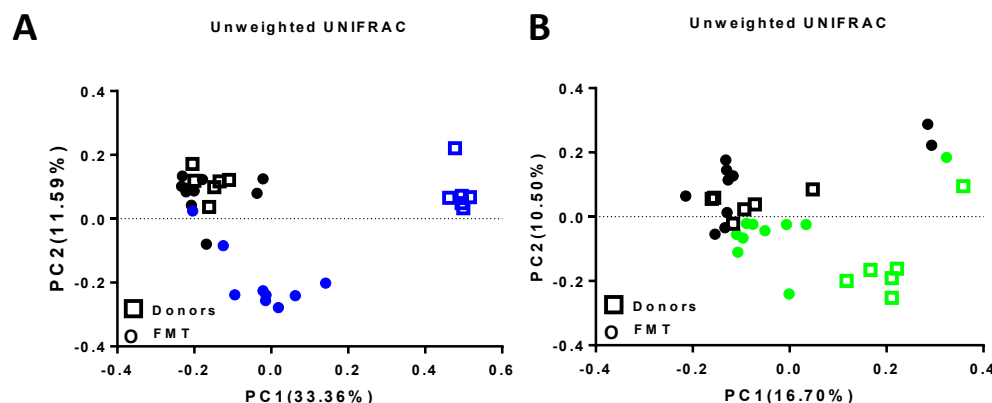


Figure 4.13 Analysis of β -diversity in donor mice and FMT mice. Principal coordinate analysis plot of microbial β -diversity between FMT-NoA group and FMT-vancomycin (A, blue) or FMT-streptomycin animals (B, green) generated using unweighted UniFrac distance matrices. Represented are data from FMT mice (O) and donor mice (\square) faecal samples collected at the end of the experiment.

4.1.4.3 Tumour immune infiltrate analysis in FMT mice

Tumour specimens from FMT-mice were analysed by IHC to characterize immune cells infiltration (Fig. 4.14). No significant differences were found in the number of CD45+ cells among groups (Fig. 4.14 A), while a significant decrease in CD3+ cells was observed in mice transplanted with faecal material from streptomycin-treated mice but not in FMT-vancomycin (Fig. 4.14 B). Similar to previous observation in antibiotic-treated mice, an increment in myeloid infiltrate in FMT-antibiotic mice tumours was observed (Fig. 4.14 C), supporting the hypothesis that antibiotic-associated microbiota composition promoted immunosuppressive tumour microenvironment. Furthermore, the intratumoral CD4+ T cells and GZMB+ cells increased upon trastuzumab treatment only in mice reconstituted with faeces from control donors (FMT-NoA) (Fig. 4.14 D-E) while the trend towards the increment of GMZB+ cells noted in FMT-streptomycin mice may explain the partial response to trastuzumab observed in these mice. These results confirm the relevance of CD4+ T lymphocytes and GZMB+ cells for trastuzumab efficacy and point out the direct involvement of the gut microbiota in the modulation of tumour immune microenvironment.

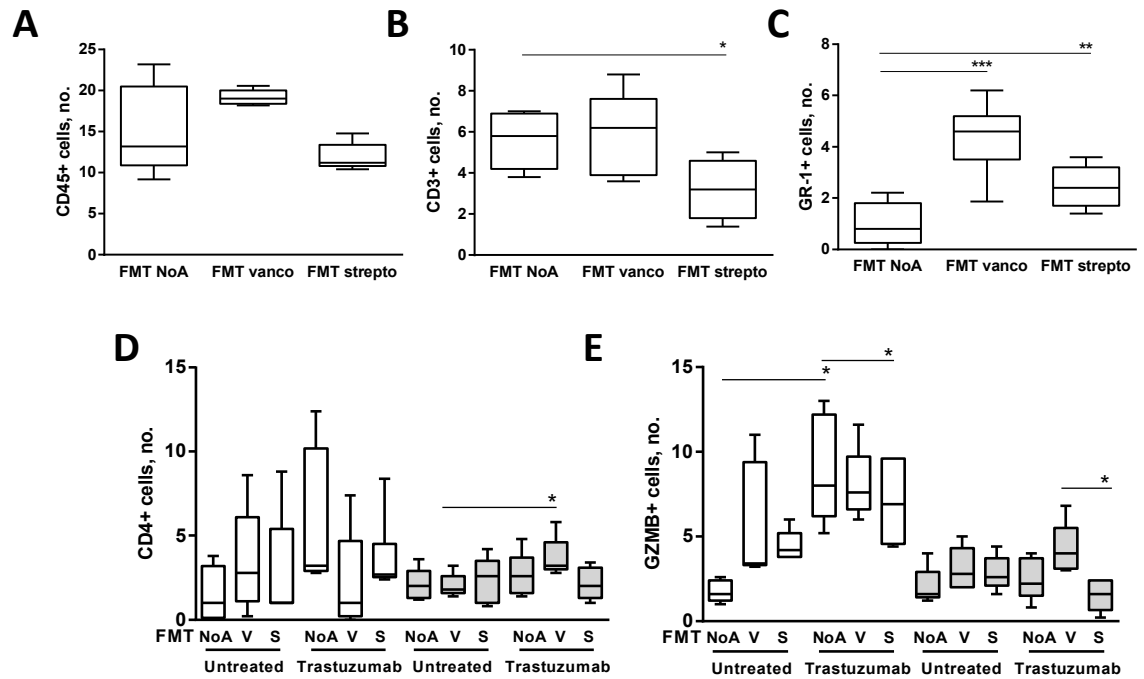


Figure 4.14 Analysis of immune infiltrate in tumours from FMT-mice. Characterization of immune infiltrate in tumours from FMT-mice. **A-C)** Count of total CD45+ (A), CD3+ cells (B), Gr-1+ cells (C). **D-F)** Intratumor and stromal staining of CD4+ T cells (C), and granzyme B (GZMB) positive (D) cells. Intratumor (white) and stromal (gray). (Data shown as box-and-whiskers, min to max) * p<0.05; **p< 0.01 and ***p<0.001 by unpaired Student's t-test.

4.1.5 Impact of gut microbiota on trastuzumab efficacy in a model of spontaneous HER2-positive breast cancer

The human HER2 receptor has been widely demonstrated to have immunogenic properties, and the possibility of HER2 to elicit an immune response cannot be ruled out. Thus, the impact of antibiotic-induced modification of gut microbiota was investigated in FVB $\Delta 16\text{HER2}$ transgenic mice ($\Delta 16\text{HER2}$), a model of spontaneous mammary gland tumorigenesis {Marchini, 2011} tolerant for the HER2 antigens, to exclude that the immunogenicity of the human HER2 receptor could have limited the interpretation of the results obtained so far in the FVB transplantable model. In this model, female mice start to develop mammary lesions from the age of eight-weeks and tumours became palpable approximately between the 12th-14th week.

To study the impact of gut microbiota alteration on tumour growth and trastuzumab efficacy, female $\Delta 16\text{HER2}$ transgenic mice were fed vancomycin in the drinking water immediately after weaning and the consequence of vancomycin administration on trastuzumab efficacy was assessed in two different treatment schedules. In first case, trastuzumab was administered to mice starting from the age of eight-weeks, when only intraepithelial lesions are present and all tumour cells rely on HER2 expression to grow (Fig. 4.15). The treatment was then continued for 10 weeks and the ability of trastuzumab to inhibit tumours development (Fig. 4.15 A) and multiplicity (Fig. 4.15 B) was similar between the two groups.

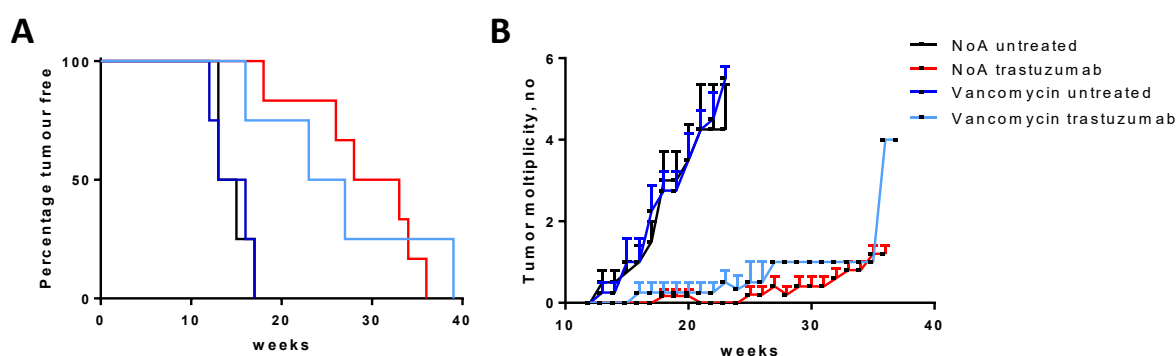


Figure 4.15 Antitumor efficacy of trastuzumab in vancomycin-treated $\Delta 16\text{HER2}$ mice. Female FVB mice were given vancomycin (200 mg/L) in the drinking water immediately after weaning. Mice were treated with trastuzumab (8 mg/kg body weight) once a week for 10 weeks. The efficacy of trastuzumab was evaluated by recording the (A) onset ($p=0.009$ by Log-rank test) and (B) multiplicity of spontaneous tumours developed in the different experimental conditions.

Further, to examine the consequences of vancomycin administration on the therapeutic efficacy of trastuzumab rather than its preventive benefit, the experiment was repeated treating mice with trastuzumab when a palpable tumour burden (almost 3 mm of diameter) was developed, a condition that is more similar to the one observed in patients. In this case, the inhibition of tumour growth was more effective in control mice as compared to vancomycin-treated mice (Fig. 4.16 A) while it was confirmed that vancomycin did not affect tumour onset (Fig. 4.16 B) and multiplicity (Fig. 4.16 C).

These results showed that the inhibition of HER2 during the early stages of tumour development is enough to block tumour growth, probably due to cancer cells addiction to HER2 signalling, independently from host conditions such as gut microbiota perturbation or tolerance to the HER2 immunogenic antigen. On the contrary, tumour cells extrinsic factors, such as gut microbiota, can impact trastuzumab therapeutic efficacy in later stage of tumour growth.

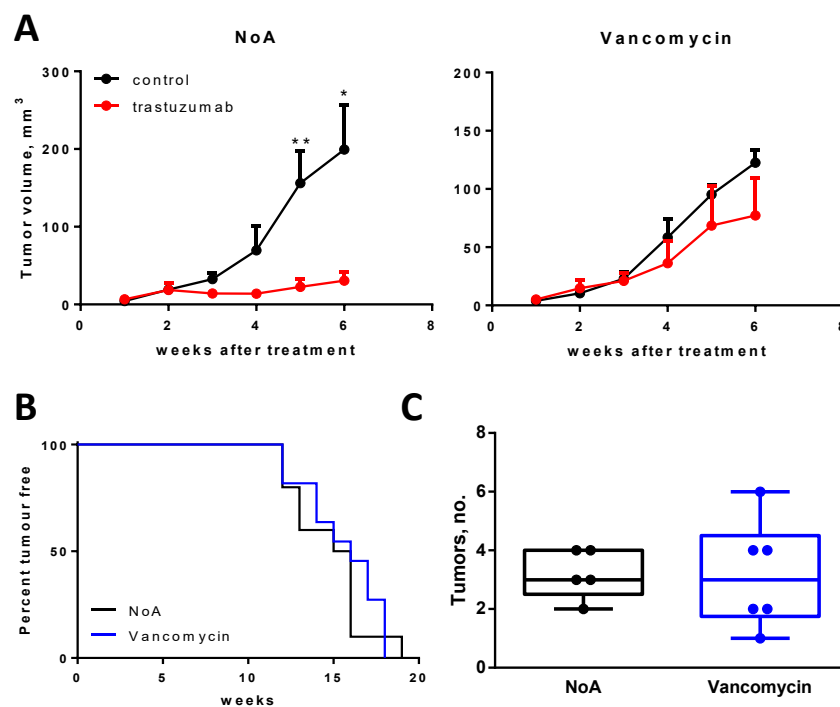


Figure 4.16 Therapeutic efficacy of trastuzumab in vancomycin-treated $\Delta 16\text{HER2}$ mice. **A)** Trastuzumab efficacy in female $\Delta 16\text{HER2}$ transgenic FVB mice treated or not with vancomycin (200 mg/L). After the first tumour onset mice were treated with 8 mg/kg of trastuzumab once a week for 6 weeks. Reported is the growth curve of the first tumour. The antibody anti-tumour efficacy was assessed by measuring tumour growth (Black, tumour growth in control mice; red, tumour growth in mice treated with trastuzumab). **B-C)** Tumour onset (B) and multiplicity (C) in female $\Delta 16\text{HER2}$ transgenic FVB mice treated with vancomycin. * $p < 0.05$ and ** $p < 0.01$ by unpaired Student's t-test.

4.1.6 Administration of lactic acid bacteria to improve trastuzumab efficacy

To explore the possibility to use lactic acid producing bacteria (LAB) to improve the antitumor efficacy of trastuzumab, two strains of LAB were used in vancomycin-treated mice. In fact, in addition to the strong decrease in the levels of total lactate found in faeces of antibiotic-treated mice, LAB are well known for their probiotic properties. *Lactococcus lactis* and *Lactobacillus paracasei* were orally administered three times a week to FVB female mice bearing MI6 tumours that were under vancomycin regimens. The treatment with the probiotic increased the inhibition of tumour growth by trastuzumab in mice under vancomycin regimen (Fig. 4.17).

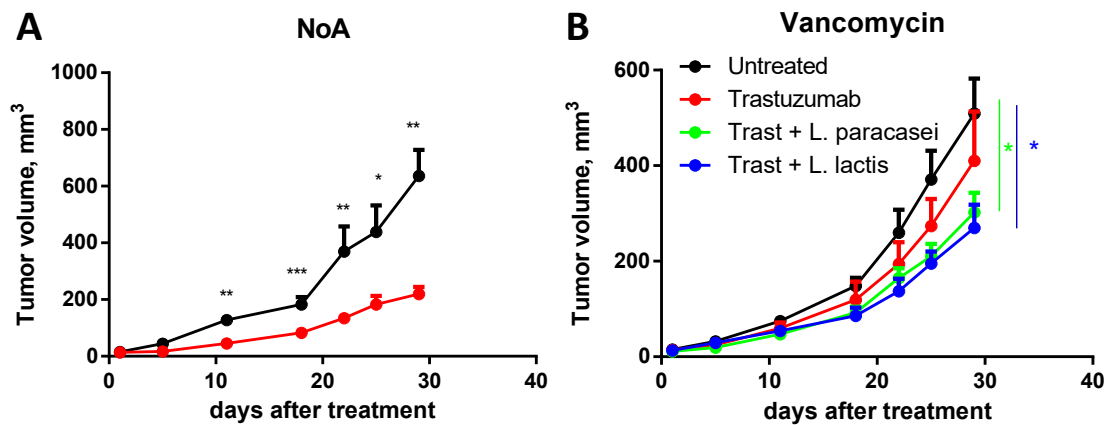


Figure 4.17 Impact of LAB on trastuzumab efficacy in vancomycin-treated mice. Vancomycin-treated FVB mice bearing MI6 tumours received 200 μ L of bacteria suspension (1×10^9 *Lactococcus lactis* or *Lactobacillus paracasei*) three times a week throughout the entire experiment. Trastuzumab was administered twice a week at a concentration of (5 mg/kg body weight) starting when tumours reached a palpable volume. Trastuzumab efficacy was measured in terms of tumour growth inhibition in control mice (A) and vancomycin-treated animals (B). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ by unpaired Student's t-test.

Based on the immunomodulatory properties of LAB, tumour immune infiltrate was analysed by flow cytometry to evaluate the influence of LAB administration on the immune-mediate activity of trastuzumab. Analysis of CD3+ positive cells and in particular CD4+ and CD8+ subpopulation did not show any significant differences among the group of treatment (Fig. 4.18 A-C). Similarly, NK cells (CD49b+) were not influenced by the manipulation of gut microbiota (Fig. 4.18 D). Intratumoral myeloid cells (CD11b+ cells)

were also analysed and besides a trend in the reduction of their frequencies in control animals upon trastuzumab administration, no significant modulations were observed (Fig. 4.18 E-F).

Further investigation should be performed to clarify the role of LAB in trastuzumab activity and elucidate the mechanisms underlying their positive influence on the mAb efficacy.

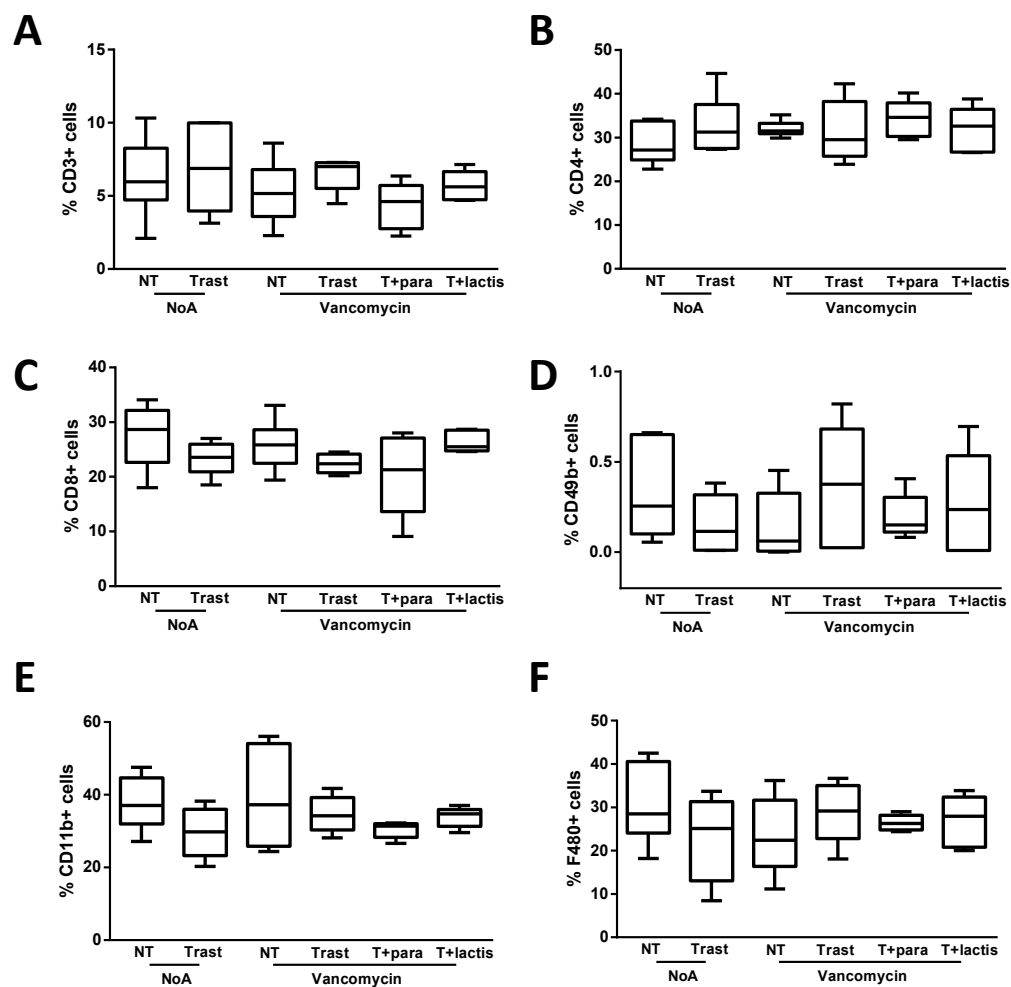


Figure 4.18 Immune characterization of tumours from LAB treated mice. Flow cytometry analysis of tumour immune infiltrate in LAB-treated mice. A-F) Frequencies of immune subpopulation are displayed: CD3+ lymphocytes T (A), CD4+ T lymphocytes in CD3+ cells (B), CD8+ T lymphocytes in CD3+ cells (C), NK cells gated on CD3 negative and CD49b + cells (D), CD11b+ myeloid cells (E), macrophages, F4/80+ cells in CD11b+ population (F).

4.2 Gut microbiota and anti-HER2 efficacy in the BALB/c murine model

To validate the results obtained so far in a different murine model of HER2-positive BC, two different cell lines were tested in BALB/c mice: D2F2/E2 and TUBO. The mouse mammary tumour line D2F2/E2 derived from a spontaneous mammary tumour grown in BALB/c mice (D2F2) and transfected to overexpress the human HER2 receptor protein {Piechoki, 2001}. TUBO cells were established from spontaneous mammary tumour in BALB/c-*neuT* transgenic mouse {Rovero, 2000} that overexpress the mutated rat neu antigen which is homologous to the human HER2 receptor. To test the sensitivity of these two cell lines to the anti-HER2 treatment, D2F2/E2 and TUBO cells were tested *in vivo* (Fig. 4.19). Mice bearing D2F2/E2 tumours received trastuzumab twice a week but no inhibition of tumour growth was observed. Conversely, the treatment with the 7.16.4 mAb, antibody specific for the rat neuT antigen, in BALB/c mice bearing TUBO tumours significantly reduced the tumour growth. Based on these results, to study the impact of antibiotic on HER2 inhibition in BALB/c mice TUBO cell line was used.

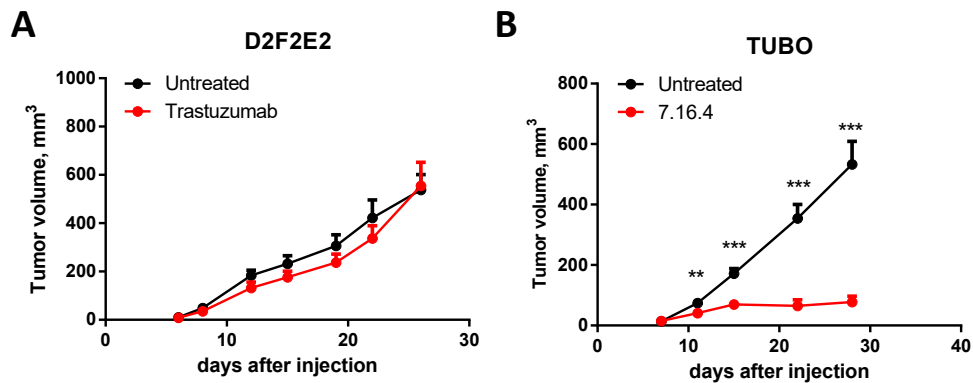


Figure 4.19 Tumour growth and anti-HER2 treatment in two BALB/c models. A) BALB/c mice bearing D2F2/E2 tumours were treated with trastuzumab (5 mg/kg body weight) twice a week starting when tumour reached a palpable volume. B) BALB/c mice bearing TUBO tumours and treated with the anti-HER2 mAb 7.16.4 (100 µg/mouse) twice a week for two weeks. The efficacy of the anti-HER2 treatment was evaluated in terms of tumour growth inhibition. Black: untreated; red: anti-HER2 treated mice. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ by unpaired Student's t-test.

4.2.1 Impact of antibiotic-induced microbiota modification on 7.16.4 mAb efficacy

4.2.1.1 Efficacy of anti-HER2 treatment in antibiotic-treated and FMT BALB/c mice

Vancomycin or streptomycin were administered in the drinking water of four-weeks old BALB/c mice for the entire duration of the experiment and TUBO cells were injected into the mammary fat pad after four weeks from the beginning of antibiotic treatment. The efficacy of the mAb 7.16.4 was assessed by treating animal twice a week for two weeks with 100 µg of mAb upon the establishment of a palpable tumour burden. In contrast to what observed in FVB mice bearing MI6 tumours, antibiotic administration did not impact the inhibition of tumour growth in 7.16.4 treated mice (Fig. 4.20 A). The same result was obtained in mice transplanted with faeces of antibiotic-treated donor mice. As observed in donor mice the anti-HER2 7.16.4 mAb equally inhibited tumour growth in both FMT-control and FMT-antibiotic mice (Fig. 4.20 B).

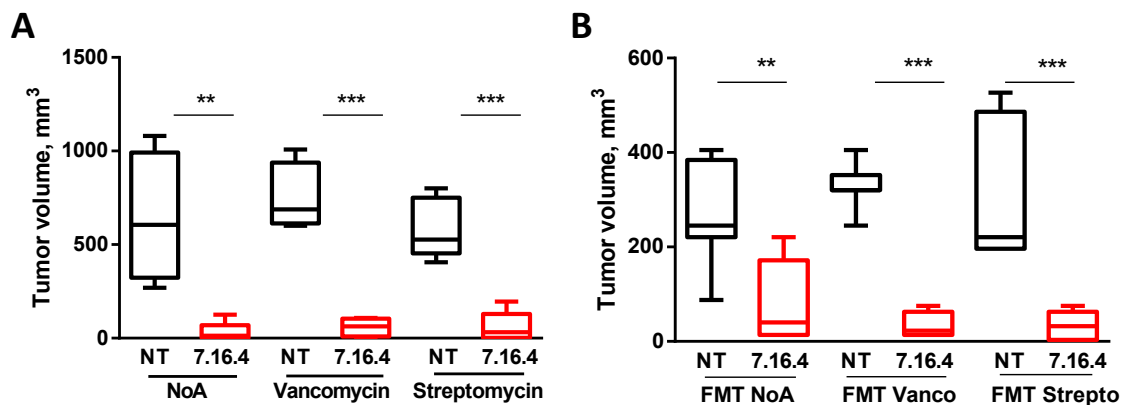


Figure 4.20 Efficacy of anti-HER2 treatment in BALB/c mice under antibiotic treatment. **A)** Tumour growth measurement in control mice (no-antibiotic, NoA) or mice under vancomycin, or streptomycin treatment (200 mg/L) **B)** Tumour growth measurement in FMT-mice. Six-weeks old BALB/c mice were treated daily for 10 consecutive days with 200 µL of ABX (vancomycin 5 mg/mL, neomycin, metronidazole and ampicillin 10 mg/mL) via oral gavage to deplete their intestinal microbiota. The gut microbiota was then reconstituted by FMT using stool suspension from A. Mice received biweekly the mAb 7.16.4 (100 µg /mouse) starting when tumour reached a palpable volume for two weeks and antitumor efficacy was assessed by measuring tumour growth. Untreated (black) and 7.16.4 mAb (red). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ by unpaired Student's t-test.

4.2.1.2 Analysis of intestinal microbiota composition

Stool samples were collected before the beginning of the anti-HER2 treatment and the content was analysed by 16S rRNA gene sequencing (Fig. 4.21). Similar to FVB mice, vancomycin had the strongest impact causing an evident decrease in the α -diversity as shown by the Chao1, Shannon, Simpson indexes (Fig. 4.21 A). As shown in figure 4.21 (Fig. 4.21 B), upon vancomycin administration a reduction in the relative abundances of the families of *Rikenellaceae*, *Porphyromonadaceae*, *Bacteroidaceae*, and *S24-7* was found concomitantly to an expansion of bacteria belonging to the phyla of *Proteobacteria* and *Verrucomicrobia*. Furthermore, a common reduction in the abundance of *Lachnospiraceae* and *Ruminococcaceae* was commonly found in the gut microbiota of mice under streptomycin and vancomycin treatment, two families that belong to the *Clostridiales* order.

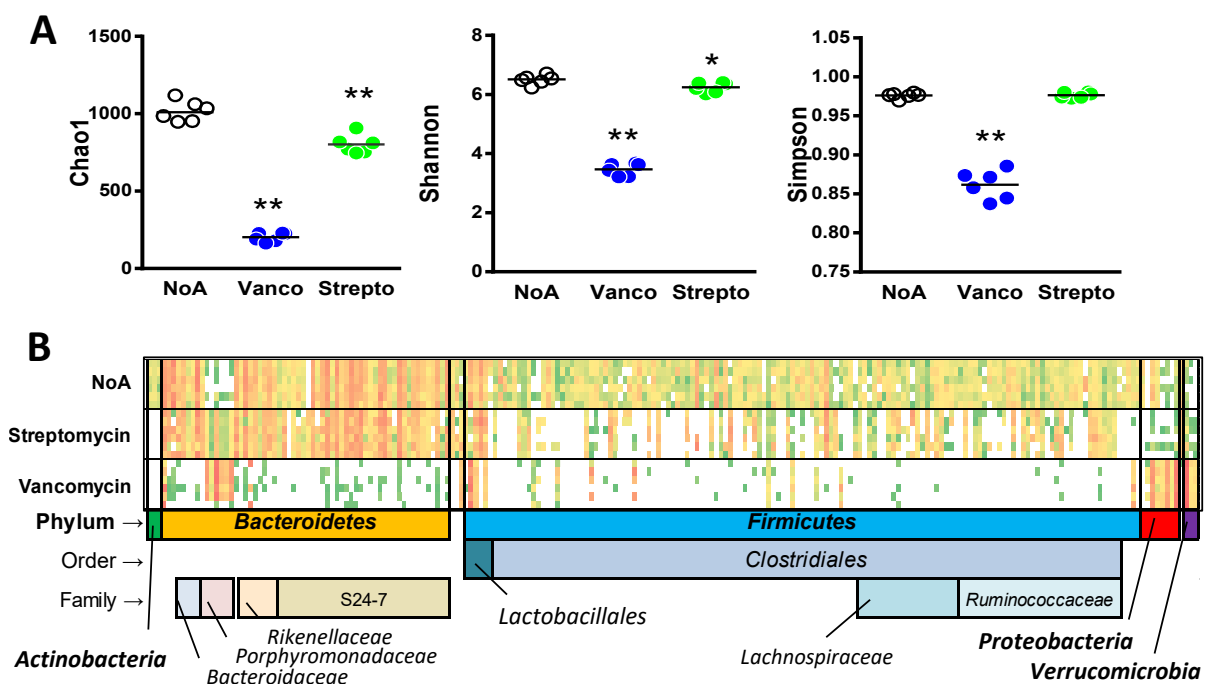


Figure 4.21 Antibiotic-induced changes in the gut microbiota of BALB/c mice. Analysis of gut microbiota composition by 16S rRNA gene sequencing of faeces from control mice (NoA) and mice fed drinking water containing vancomycin, or streptomycin (200 mg/L). **A-B**) Microbial α -diversity between NoA and vancomycin or streptomycin treated mice calculated by Chao1, Shannon and Simpson indexes. **B**) Comparison of bacterial genus abundance between the three groups of treatment. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired Student's t-test.

4.2.2 Influence of gut microbiota depletion on tumour immune infiltrate and anti-HER2 treatment

4.2.2.1 Characterization of tumour immune infiltrate in BALB/c mice

During my six-months visit to the laboratory of Dr. Romina Goldszmid at the National Institute of Health (NIH), Bethesda Maryland, the immune infiltrate of TUBO tumours was analysed by flow cytometry in mice treated with vancomycin or ABX. Vancomycin (200 mg/L) was administered in the drinking water of mice for 4 weeks before TUBO cells injection into the mammary fat pad, in parallel a different group of mice were treated with the antibiotic cocktail routinely used to study the impact of gut microbiota on antitumor immunity in the hosting laboratory: vancomycin (500 mg/L) neomycin (700 mg/L) and Primaxin (500 mg/L) {Iida, 2013}. Tumours were harvested after two weeks from injection and single cell suspension were analysed by flow cytometry (Fig. 4.22) in order to study the priming effect of commensal bacteria on tumour immunity. No significant differences in tumour volume were observed among control and antibiotic (vancomycin and ABX) treated mice (Fig. 4.22 A), while ABX but not vancomycin caused a decrease in the frequency of CD45+ cells within the tumours (Fig. 4.22 B).

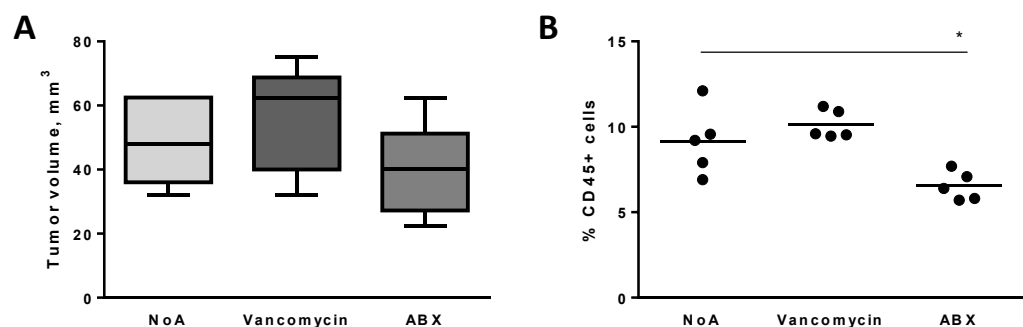


Figure 4.22 Analysis of the tumour immune infiltrate in antibiotic-treated BALB/c mice. Antibiotics were dissolved in the drinking water: vancomycin was given at a concentration of 200 mg/L, while the ABX cocktail contained vancomycin (500 mg/L), neomycin (700 mg/L) and Primaxin (500 mg/L). After four weeks, 5×10^5 TUBO cells were injected into the mammary fat pad of mice and let grow for two weeks. **A)** Tumour volumes at the time of the harvest. **B)** Frequencies of CD45+ cells in live cells as analysed by flow cytometry. * $p < 0.05$ by Student's t-test.

To characterize the immune infiltrate, markers of the lymphoid and myeloid lineage were used, and immune subpopulation were selected in CD45⁺ live cells. No differences were found in the tumour microenvironment of mice whose intestinal microbiota was altered by the treatment with vancomycin while evident modifications were observed in the ABX group. The disruption of the intestinal microbiota resulted in altered infiltration of both lymphoid (Fig. 4.23) and myeloid (Fig. 4.24) cells in the tumours. The frequencies of T lymphocytes (TCR β ⁺ cells) and $\gamma\delta$ T cells were decreased, and the ABX treatment associated with a decreased frequency of CD4⁺ T cells within the tumour, in particular a prevalence of naïve CD4⁺ T cells was observed in ABX-treated mice as compared to the control (Fig. 4.23 A-C) and vancomycin groups, characterized by a higher amount of memory T cells. This evidence was supported by a reduced percentage of CD44⁺ CD62L⁻ cells in the tumour of ABX mice (Fig. 4.23 D) and the lower CD44 but higher CD62L MFI in this group than in the controls (Fig. 4.23 E-F). No significant differences were observed in CD8⁺ T cells and B (CD3^{neg} CD19⁺) cells (Fig. 4.23 G-H). The treatment with the ABX also impaired tumour infiltration by NK (CD49b⁺NKp46⁺) cells (Fig. 4.23 I).

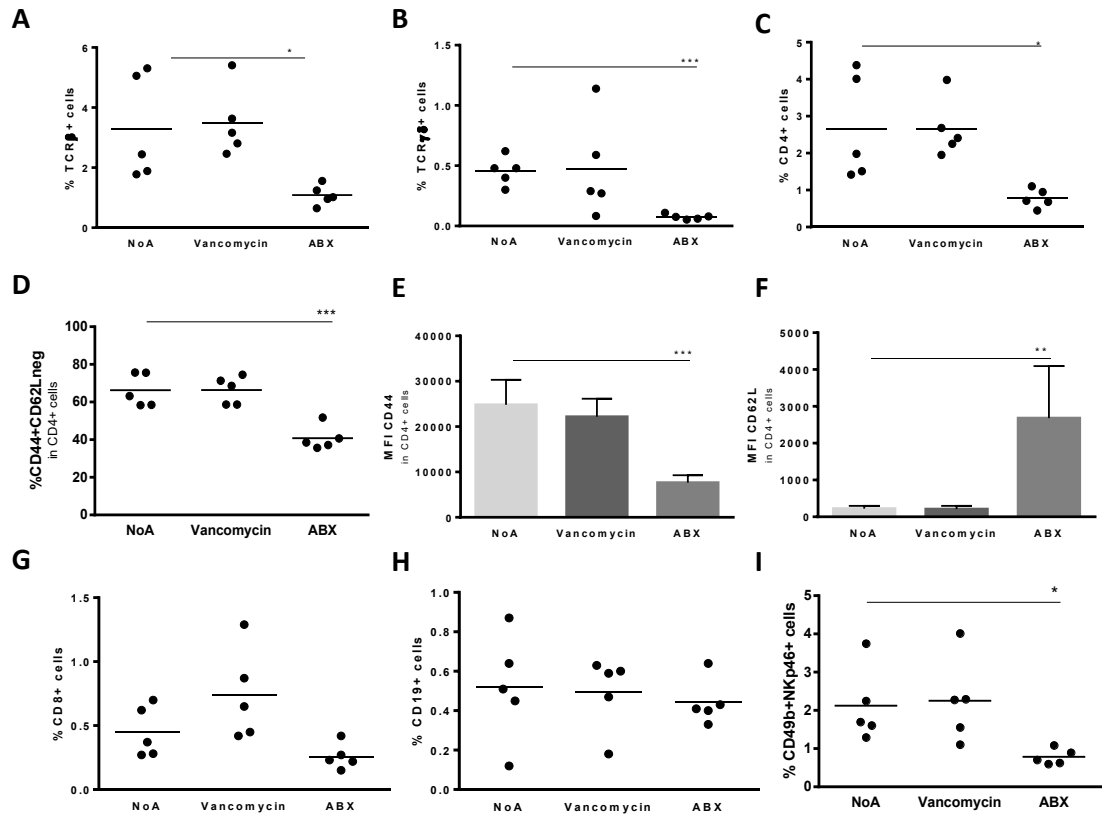


Figure 4.23 Characterization of lymphoid tumour infiltrate by flow cytometry. A-D,G-I) Frequency of lymphoid cells gated on CD45⁺ live cells: T lymphocytes (A); $\gamma\delta$ T cells (B); CD4⁺ T cells (C); memory T CD4⁺ cells (CD44⁺CD62L^{neg} in CD4⁺) (D); CD8⁺ T lymphocytes; CD19⁺ B lymphocytes (H); NK cells (CD49b⁺NKp46⁺) (I). E-F) Median fluorescence intensity of CD44 and CD62L expression in CD4⁺ T cells. Cell frequency is represented as the percentage of positive cells in the CD45⁺ population. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by unpaired Student's t test.

Modulation of the myeloid compartment was also found as regard to the percentage of neutrophils and monocytes infiltration. The depletion of intestinal microbiota was associated with an increase in the frequency of neutrophils (Ly6G⁺ cells) in the tumour and a concomitant reduction in the percentage and number of infiltrating monocytes (Ly6C^{high} cells) (Fig. 4.24 A-B). The treatment with the antibiotics also contributed to create an immunosuppressive tumour microenvironment by promoting the infiltration of M2-like macrophages, as suggested by the increased frequency of the F4/80⁺CD206⁺ subpopulation (Fig. 4.24 C). In addition, tumours from the ABX group were characterized by a reduced infiltration of DCs (Fig. 4.24 D).

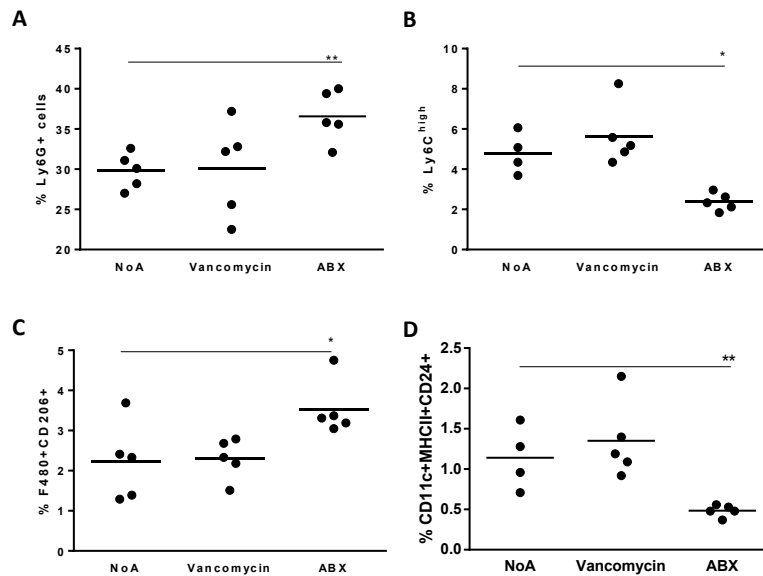


Figure 4.24 Characterization of myeloid tumour infiltrate by flow cytometry. A-D) Frequency neutrophils (Ly6G+ cells) (A); monocytes (Ly6C^{high}) (B); M2-like macrophages (F4/80+CD206+) (C); dendritic cells (DCs, CD11c+MHCII+CD24+) (D). Cell frequency is represented as the percentage of positive cells in the CD45+ population. *p<0.05; **p<0.01 by unpaired Student's t test.

4.2.2.2 Efficacy of the anti-HER2 treatment in ABX-treated mice

Based on the different impact of ABX and vancomycin on tumour immune cells, and the promotion of an immunosuppressive microenvironment, the efficacy of the mAb 7.16.4 was investigated in this model. The ABX cocktail containing vancomycin (500 mg/L), neomycin (700 mg/L) and ampicillin (1 g/L) to deplete intestinal microbiota was administered in the drinking water of mice. After four weeks of ABX treatment, TUBO cells were injected into the mammary fat pad as already described and when tumours reached a palpable volume mice received 100 µg of the 7.16.4 mAb twice a week for two weeks. In line with previous observation in BALB/c animals, ABX had no consequences on tumour growth and, as found in single-antibiotic experiment, the efficacy of the mAb 7.16.4 was not affected by the depletion of intestinal microbiota in mice (Fig. 4.25).

These results suggest that TUBO cells depend on the oncogenic signalling of HER2/neu to grow, and its inhibition with the anti-HER2 mAb 7.16.4 is sufficient to

inhibit tumour growth independently of the gut microbiota composition/depletion and its modulation of the tumour immune microenvironment.

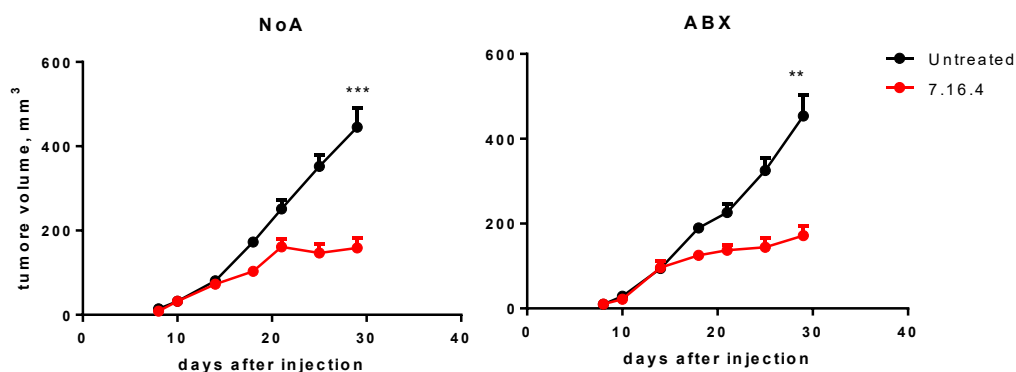


Figure 4.25 Anti-HER2 mAb efficacy in ABX-treated mice. BALB/c mice were treated with antibiotic cocktail (vancomycin 500 mg/L, neomycin 700 mg/L and ampicillin 1 g/L) starting from four-weeks of age. After 28 days of treatment TUBO cells were injected into the mammary fat pad and the treatment with 7.16.4 was started when tumour reached a palpable volume. mAb was administered twice a week for two weeks. ** $p < 0.01$; *** $p < 0.001$ by unpaired Student's t test.

4.3 Gut microbiota and trastuzumab-based therapy in HER2-positive BC patients

4.3.1 Trastuzumab benefit and gut microbiota composition

4.3.1.1 Clinical characteristics of HER2-positive BC patients

To assess the clinical relevance of our findings, the association between intestinal microbiota composition and trastuzumab benefit was investigated in HER2-positive BC patients treated with neoadjuvant trastuzumab-based chemotherapy. The study was performed in collaboration with Dr. Fabio Corsi from the Dept. of Breast Surgery at the Istituti Clinici Scientifici Maugeri Spa-SB of Pavia between 2017 and 2019. Women received four cycle of cyclophosphamide and anthracycline-based (AC) treatment followed by four to six cycles of taxanes and trastuzumab (TH) before surgery, two of them received TH since the beginning for six cycles. Tumour biopsies and faecal samples were collected from patients at diagnosis and before the beginning of trastuzumab treatment respectively (Fig. 4.26).

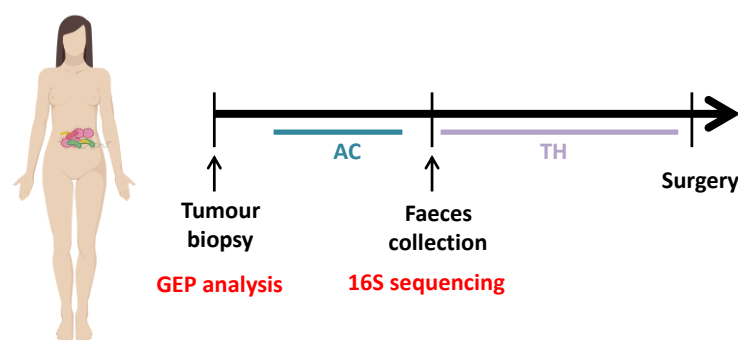


Figure 4.26 Samples collection from HER2-positive BC patients. Tumour biopsy and stool samples were collected at diagnosis and before trastuzumab treatment respectively. Gene expression profile (GEP) was performed on available tumours and the gut microbiota composition was analysed by 16S rRNA gene sequencing.

Patients who achieved pCR and were considered responsive (R) while those with breast residual disease at the time of the surgery were classified as non-responders (NR), clinical information of patients enrolled in the study are summarized in table 4.1. No major differences in patients characteristics were observed in R versus NR except for the lymph nodes status (N) with 43% versus 10% of N+ patients in NR and R, respectively (Table 4.2).

Table 4.1 Clinical information of the patients enrolled in the study.

Patient ID	Response to treatment	Age at diagnoses	Histotype	Grade	Lymph node status	ER status	PR status	HER2 status	Ki67 % positive cells	PAM50	Microbiota cluster	Neoadjuvant treatment	Tumour size by ultrasound before treatment (mm)	Tumour size by histological evaluation at surgery (mm)	Antibiotics usage before treatment (months)
1	R	51	IDC	III	POS	NEG	NEG	3+	70	HER2-E	2	AC+TH	30	0	None
3	R	41	IDC	II	POS	POS	POS	3+	30	HER2-E	2	AC+TH	18	0	Fosfomycin (<3months)
5	R	47	IDC	II	POS	NEG	NEG	3+	30	HER2-E	2	AC+TH	15	0	None
6	R	55	IDC	II	POS	POS	POS	3+	20	HER2-E	2	AC+TH	60	0	None
9	NR	72	IDC	III	POS	POS	POS	3+	30	HER2-E	1	AC+TH	24	20	None
10	R	36	IDC	III	POS	POS	POS	3+	30	Basal	2	AC+TH	20	0	None
12	R	66	IDC	III	POS	POS	POS	3+	50	HER2-E	1	AC+TH	30	0	na
13	R	46	IDC	III	POS	POS	POS	2+ amplified	70	HER2-E	2	AC+TH	60	0	Amoxicillin (<3months)
14	NR	41	IDC	II	NEG	POS	POS	3+	15	Luminal B	1	AC+TH	20	4	None
15	NR	59	IDC	II	POS	POS	POS	3+	40	HER2-E	1	AC+TH	20	2	na
16	NR	51	IDC	II	POS	NEG	NEG	3+	80	na	1	AC+TH	42	10	None
18	NR	59	IDC	II	NEG	POS	POS	3+	40	HER2-E	2	AC+TH	27	5	None
21	R	45	IDC	III	POS	NEG	POS	3+	50	Basal	2	AC+TH	8	0	None
22	NR	76	ILC	III	NEG	POS	POS	2+ amplified	30	HER2-E	1	TH	20	1	None
23	NR	70	IDC	III	NEG	POS	POS	3+	25	na	2	AC+TH	25	12	Amoxicillin (>3months)
24	R	60	IDC	III	POS	NEG	NEG	3+	60	na	2	AC+TH	35	0	None
26	R	57	IDC	II	POS	POS	NEG	3+	25	na	2	TH	30	0	None
27	R	71	IDC	II	NEG	POS	NEG	3+	10	na	2	AC+TH	15	0	None

ER, oestrogen receptor (>1% positive cells); PR, progesterone receptor (>1% positive cells); R, responsive; NR, non-responsive; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; HER2-E, HER2-enriched; AC, anthracycline and cyclophosphamide; TH, taxanes and trastuzumab; na, not available.

Table 4.2 Clinicopathological variables of the analysed patients

	pCR	NR	p-value*	Cluster 1	Cluster 2	p-value*
Age (years)			0.1467 [‡]			0.2227 [‡]
Median (range)	51 (41-71)	59 (41-76)		62 (41-76)	53 (41-71)	
Tumour size			1.00			1.00
≤ 2 cm	5 (45%)	3 (43%)		3 (50%)	5 (42%)	
>2 cm	6 (55%)	4 (57%)		3 (50%)	7 (58%)	
Lymph node status			0.0474			1.00
Negative (N-)	1 (16%)	4 (57%)		2 (33%)	3 (25%)	
Positive (N+)	10 (84%)	3 (43%)		4 (67%)	9 (75%)	
ER status			1.00			1.00
Negative	4 (47%)	2 (29%)		2 (33%)	4 (33%)	
Positive	7 (53%)	5 (71%)		4 (67%)	8 (67%)	
PR status			0.3156			0.60
Negative	5 (45%)	1 (14%)		1 (17%)	5 (42%)	
Positive	6 (55%)	6 (86%)		5 (83%)	7 (58%)	
Grade			1.00			1.00
I and II	5 (45%)	4 (57%)		3 (60%)	6 (50%)	
III	6 (50%)	3 (43%)		2 (40%)	6 (50%)	
PAM50						
HER2-E	6 (75%)	4 (80%)	1.00	4 (80%)	6 (75%)	1.00
Others	2 (25%)	1 (20%)		1 (20%)	2 (25%)	

ER, oestrogen receptor; PR, progesterone receptor; pCR, pathological complete response; NR, non-responsive; HER2-E, HER2-enriched. *, *p*-value of the Fisher exact test. [‡], *p*-value of the Mann-Whitney test

4.3.1.2 Analysis of the gut microbiota composition in HER2-positive BC patients

To evaluate whether commensal bacterial composition associated with clinical benefit of trastuzumab the DNA was extracted from faecal samples of 18 HER2-positive BC patients collected before the beginning of trastuzumab treatment and the 16S rRNA gene was profiled. The α -diversity between patients was analysed by using Chao1, Shannon and Simpson indexes and a trend towards significant higher diversity in R compared to NR patients was observed (Fig. 4.27 A). The PCoA of intestinal ecology β -diversity revealed a clustering effect between R and NR patients showing a possible association between microbiota characteristics and response to trastuzumab (Fig. 4.27 B).

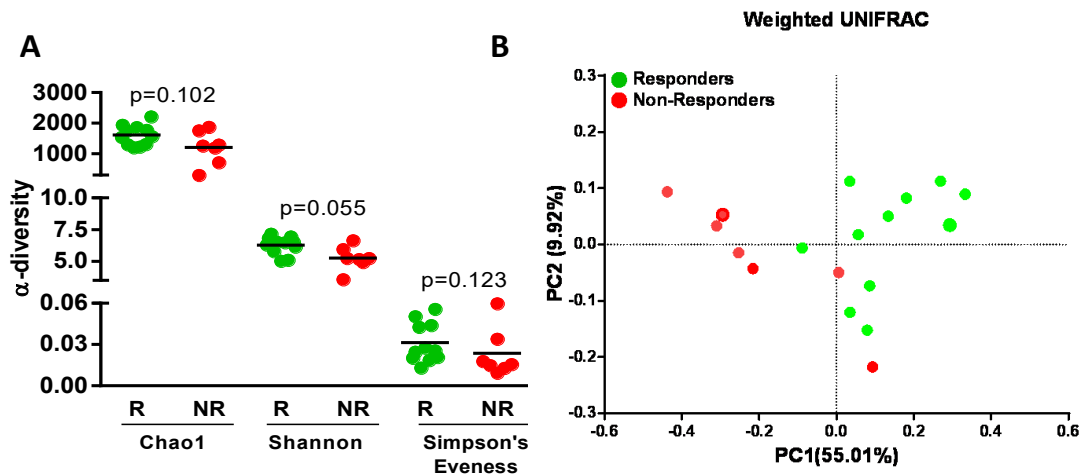


Figure 4.27 Metagenomic analysis of faecal samples from HER2+ BC patients. The composition of the gut microbiota was analysed in 18 HER2-positive BC patients treated with neoadjuvant trastuzumab-based chemotherapy. **A)** Analysis of α -diversity between R (n=11) and NR (n=7). Microbial α -diversity was calculated by different indexes (i.e. Chao1, Shannon and Simpson indexes). **B)** Analysis of β -diversity between R (green) and NR (red). Principal coordinate analysis for microbial was generated using weighted UNIFRAC distance.

To identify differentially abundant bacteria between patients who benefitted or not from trastuzumab treatment, data obtained from the 16S rRNA gene profiling was analyzed by LEfSe. Patients who achieved pCR were characterized by a microbiota enriched in bacteria from the *Clostridiales* taxonomic orders while *Bacteroidales* were more abundant in NR than R. Specifically, members from the taxonomic families *Lachnospiraceae*, *Turicibacteriaceae*, *Coriobacteriaceae* and *Prevotellaceae* were less represented in NR patients, as observed in animals upon antibiotic treatment, suggesting their possible role in favouring the response to trastuzumab (Fig. 4.28 A-B).

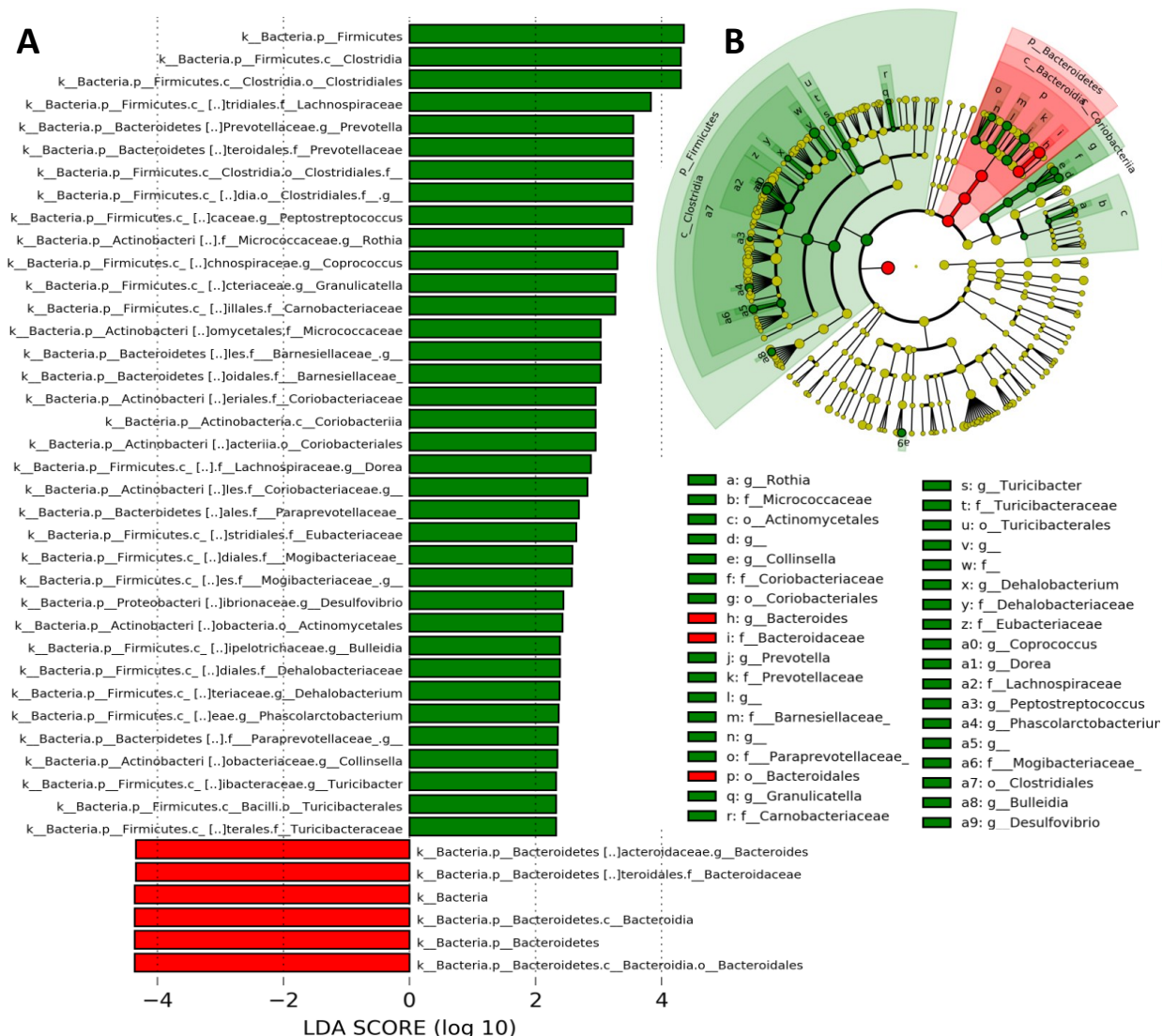


Figure 4.28 Differences in bacterial community according to patients response. A) Linear discriminant analysis (LDA) scores computed for differentially abundant taxa in the faecal microbiomes of R (green) and NR (red). Length indicates effect size associated with a taxa. **B)** Taxonomic cladogram from LEfSe showing differences in faecal taxa. Dot size is proportional to the abundance of the taxa.

When the content of SCFAs was analysed in stool samples from 11 HER2+ BC patients (7 R and 4 NR), a trend towards a reduction of acetate, propionate and butyrate, but not lactate, was observed in NR patients despite the low number of samples and the high variability detected (Fig. 4.29).

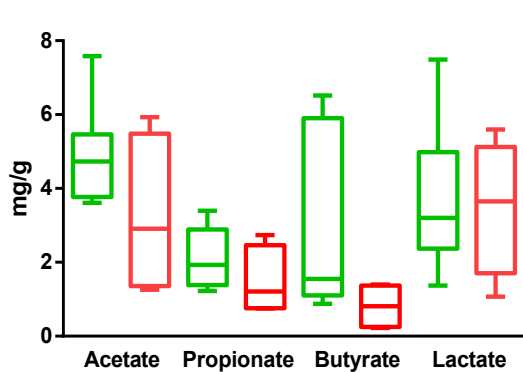


Figure 4.29 SCFAs and lactate quantification in HER2-positive BC patients. Products of bacteria metabolism were analysed in faecal samples collected before trastuzumab treatment in R (green) and NR (red). The analysis was performed by ultraperformance liquid- chromatography-high resolution-mass-spectrometry analysis (UPLC-HR-MS) for SCFAs and by enzymatic kit for lactate.

4.3.1.3 Causal role of the gut microbiota in HER2-positive BC patients treatment.

The causal link between a “favourable” gut microbiota and the response to trastuzumab was assessed by transferring faecal material from R (n=6) and NR (n=4) patients into recipient mice (Fig. 4.30 A). Notably, R-FMT mice benefitted from the anti-HER2 treatment in contrast to NR-FMT mice confirming the direct involvement of commensal bacteria in trastuzumab efficacy (Fig. 4.30 B).

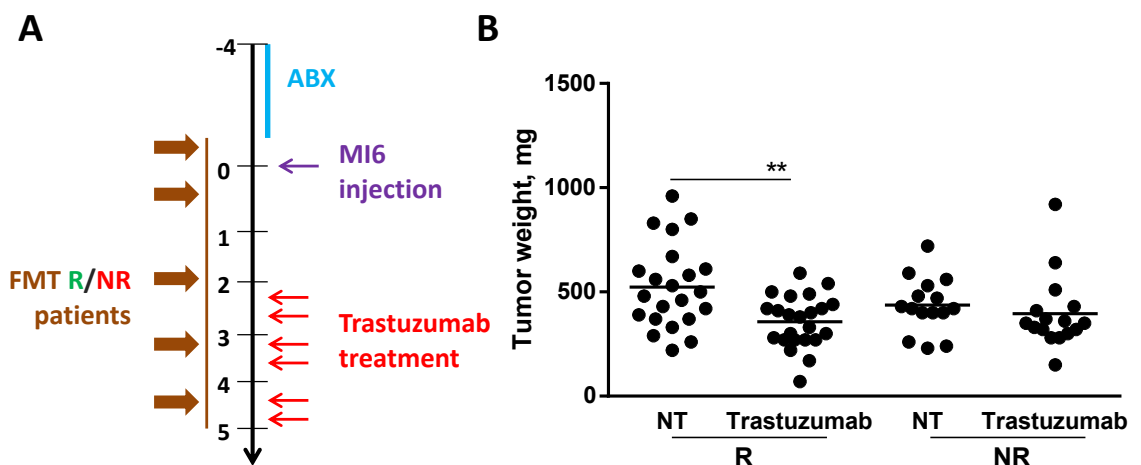


Figure 4.30 Human commensal bacteria modulate trastuzumab antitumor efficacy in mice. A) FVB mice were depleted from their intestinal microbiota using ABX cocktail and were then gavages with faecal material from responsive patients (R, n=6) and non-responders (NR, n=4). Faecal material was transplanted 48h before and after tumour cells injection and then every 10 days for a total of 5 FMT (7-8 mice/patients were used). Trastuzumab (5 mg/kg body weight) was administered twice a week for three weeks starting when tumours reached palpable volumes B-C) Tumour weight measurement evaluated at the end of the experiment (B). **p<0.01 by unpaired Student’s t test.

4.3.2 Predictive potential of commensal bacteria composition in neoadjuvant treated HER2-positive BC patients

To investigate the potential use of the gut microbiota as a predictive biomarker, an unsupervised clustering analysis was applied to patient's β -diversity and two clusters separate patients according to trastuzumab response (silhouette=0.33) (Fig. 4.31 A): 71% (5/7) of NR patients segregated in cluster 1 and 91% (10/11) of R gathered together in cluster 2. The two clusters significantly associated with response to trastuzumab as analysed by Fisher's exact test ($p=0.0128$). Based on the relevance of tumour intrinsic characteristics in discriminating patients sensitive or not to trastuzumab, the PAM50 subtype predictor was applied to GEP of tumour core biopsies available for 13 out of 18 patients. The HER2-enriched tumours, defined as the most responsive to trastuzumab, were found in both the microbiota clusters (4/10 in cluster 1 and 6/10 in cluster 2) (Fig. 4.31 and table 4.2), indicating that gut microbiota may have the potential to join further information to HER2-enriched BC subtype in identifying R or NR patients.

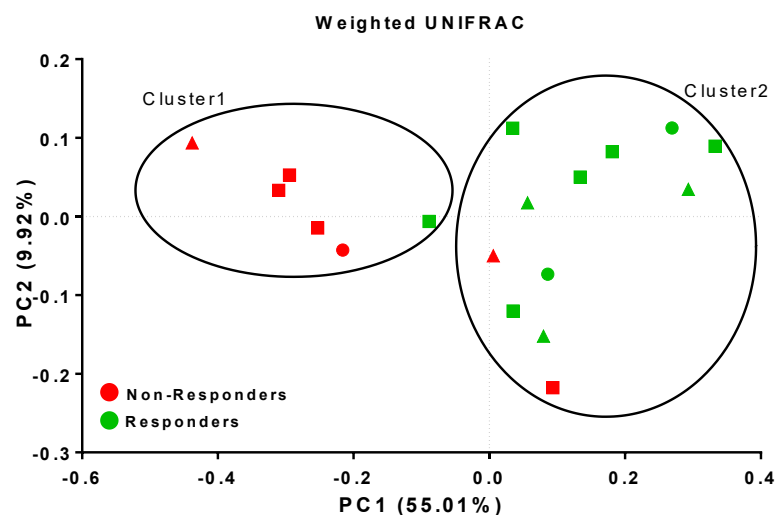


Figure 4.31 Association between gut microbiota and response to the anti-HER2 treatment. The unsupervised clustering was performed by applying the JSD algorithm to the β -diversity analysis derived from weighted UniFrac algorithm: Cluster 1 (5 NR + 1 R) and Cluster 2 (2 NR + 10 R) associated with the response to trastuzumab as evaluated by Fisher's exact test ($p=0.0128$). Tumour intrinsic subtypes defined by PAM50 molecular classifier are displayed as (\square) HER2-enriched tumours, (\circ) basal tumours, (Δ) tumours whose GEP was not available.

Chapter 5: DISCUSSION

The introduction of trastuzumab (Herceptin) in the clinical use has largely improved the clinical outcome of early, advanced and metastatic HER2-positive patients. Nonetheless, a high number of women receiving trastuzumab do not benefit from this treatment and the reasons underlying the heterogeneous response observed in patients still need to be fully comprehend. Moreover, studies focused on the development of response predictors that are based on tumour characteristics still miss a high number of patients that do not benefit from trastuzumab. This evidence and the fact that even the double-block of the HER2 receptor did not completely remove the presence of resistant patients, moved researchers to consider extrinsic tumour factors that could be involved in trastuzumab efficacy. Preclinical and clinical evidence unravelled the influence of the gut microbiota on anticancer therapies that rely on immune activation for their activity, and since trastuzumab can be considered an immunomodulatory drug, in this project, the impact of commensal bacteria on its antitumor activity was investigated in *in vivo* experiments using models of HER2-positive breast cancer and in patients that received neoadjuvant trastuzumab-based therapy.

Firstly, the involvement of gut commensal bacteria in the therapeutic efficacy of trastuzumab was unravelled by changing the intestinal bacteria ecosystem by the administration of vancomycin and streptomycin that resulted in the complete abrogation of tumour growth inhibition by trastuzumab in FVB mice. So far, the manipulation of the gut microbiota by antibiotics, has been widely used by researchers to demonstrate the relevance of commensals in the anticancer efficacy of chemotherapeutic drugs such as doxorubicin, and cyclophosphamide {Viaud, 2015} and of immunotherapy (i.e. anti-PD1 {Routy, 2018a}), anti-CTLA4 {Vétizou, 2015} and anti-IL10R {Iida, 2013}). Despite the different mechanisms of action of vancomycin, mainly directed against Gram-positive bacteria {Hammes, 1974} and streptomycin, a broad spectrum protein synthesis inhibitor {Demirci, 2013}, the two antibiotics equally inhibited trastuzumab efficacy suggesting that

the gut community structure significantly affected the response to therapy. While vancomycin robustly reduced α -diversity and caused a wide decrease in the abundances of bacterial taxa belonging to *Bacteroidetes* and *Firmicutes* taxonomic phylum with a parallel over-representation of *Proteobacteria* and *Verrucomicrobia*, streptomycin mainly affected the relative abundance of bacteria in the *Firmicutes* phylum. Within the phylum *Firmicutes*, both antibiotics determined the reduction of numerous taxa belonging to the order of *Clostridiales*, and particularly to the *Lachnospiraceae* family and SCFA-producing taxa like *Ruminococcaceae* and *Lachnospiraceae* (together with the *Odoribacter* genus in the *Bacteroidales* taxonomic order) that can explain the decrease in the level of butyrate, propionate and acetate observed in antibiotic treated mice. These products of bacterial fermentation are usually exploited by intestinal epithelial cells as a source of ATP but they can also have important immunomodulatory function as for instance butyrate, by activating the GCPRs on IECs and immune cells, favours the maintenance of the gut barrier inhibiting the inflammatory responses in human monocytes {Parada Venegas, 2019}. SCFAs represent the connection between intestinal ecology and systemic immunity and may be the key to overcome the difficulties related to the microbiota therapeutic intervention being exploited to improve anticancer treatment efficacy in future. To investigate these aspects, SCFAs levels were quantified in the blood of mice and their amount resulted to be lower in antibiotic-treated animals than in controls indicating that gut conditions can be reflected systemically even though we are aware that these results should be considered with caution due to the <LOQ quality of our samples.

When the tumour immune microenvironment was analysed, major differences were found upon trastuzumab administration but not at basal levels, in fact trastuzumab induced a spatial redistribution between stroma and tumour nests of CD4⁺ T lymphocytes and GZMB⁺ cells in tumours of control animals but not in tumours of mice under vancomycin or streptomycin treatment. Coherently, the relevance of T lymphocytes and NK cells was

supported by the analysis of the expression levels of *Il12a* and *Il15*, two proinflammatory cytokines required for proliferation and activation of Th1 lymphocytes and NK cells, that were found to be higher in control than in vancomycin or streptomycin mice treated with trastuzumab. These findings are in line with published data by which alteration of the gut microbiota have been reported to negatively impact immune cells activation in response to cancer treatment (i.e. CpG oligonucleotide and oxaliplatin {Iida, 2013} or anti-CTLA4 {Vétizou, 2015}). Moreover, the analysis of the intratumoral cytokines expression showed higher levels of *il15* in vancomycin- and streptomycin-treated animals than control. IL-15 is produced by macrophages and it is involved in the activation of NK cells and CD8⁺ T cells with the potential to enhance the antitumor activity of trastuzumab when used in combination {Wege, 2017}. However, the continuous stimulation with IL15 can cause NK cells exhaustion {Felices, 2018} that may explain why mice under antibiotic regimen did not benefit from trastuzumab treatment. Based on higher number of Gr-1 and Arg1 positive cells and on higher *tgfb1* expression levels in tumours from antibiotic-treated than control mice, we can hypothesise the development of a suppressive immune microenvironment in antibiotic-treated mice. Starting from these observations, further experiments will be essential to deeper investigate the mechanisms by which trastuzumab is influenced by commensal bacteria and whether the modulation of tumour microenvironment directly depends on gut microbiota.

Surprisingly, different results were obtained in a second transplantable model of HER2-positive breast cancer in BALB/c mice: vancomycin and streptomycin administration did not impact the efficacy of the anti-HER2 mAbs 7.16.4 against the growth of TUBO tumours although the antibiotic-induced modifications of the gut microbiota were similar to the one observed in FVB mice. Further investigation in the BALB/c model revealed that the depletion of the intestinal microbiota by ABX had a strong consequences on the tumour immune infiltrate lowering frequencies and numbers of

immune cells such as NK cells, CD4⁺ T cells, DCs and macrophages, accompanied by an increase in neutrophils and CD206⁺ macrophages with potential suppressive properties. Nonetheless, even the establishment of a pro-tumorigenic immune phenotype was not enough to affect trastuzumab antitumor efficacy suggesting that in TUBO cells, which overexpressed the mutated human homologue HER2/*neu* receptor, the inhibition of HER2 signalling is sufficient to inhibit tumour growth in an immune-/microbiota-independent mechanism. Likely, the gut microbiota plays a relevant role especially in those tumour in which the efficacy of the anti-HER2 treatment relies the most on the engagement of immune cells to inhibit tumour growth, while it has a marginal, or even no role, in those tumours completely dependent on the HER2 intracellular signalling. In support to this hypothesis, the modification of gut microbiota by vancomycin did not affect the efficacy of trastuzumab FVB Δ 16HER2 transgenic mice when the treatment was started before tumour onset when only intraepithelial lesions are present and the proliferation of tumour cells is dependent from the Δ 16HER2 receptor. The vancomycin-impairment of trastuzumab efficacy, was only observed when trastuzumab was administered in Δ 16HER2 mice with already established tumours supporting the role of gut microbiota in the immune-mediated efficacy of trastuzumab. Furthermore, vancomycin treatment had no consequences on tumour onset and multiplicity in FVB Δ 16HER2 mice differently from previously published results in which antibiotic administration associated with increased development of mammary gland tumours {Rossini, 2006} in Proto-*neu* transgenic mice.

The consistency between the results obtained in both spontaneous and transplantable FVB model suggests that the interpretation of the data was not limited by the immunogenic potential of HER2, in fact an immune response elicited against the HER2 antigen in the transplantable tumour model could have concealed the influence of gut microbiota on the immune-mediated activity of trastuzumab.

It is possible that antibiotic treatment can have a direct cytotoxic effect on tumours as some of them are reported to inhibit proliferation in human mesenchymal stem cells {Bariteau, 2018} osteoblasts {Eder, 2016} and glial cells {Sofian, 2012}. Thus, although vancomycin and streptomycin are characterized by a poor adsorption in the intestine, a systemic effect of the antibiotic on tumour cells cannot be ruled out. However, the analysis of HER2 expression and modification of the tumour microenvironment, excluded the negative impact of antibiotics on trastuzumab distribution within the tumours and on its binding to HER2, two possible reasons for a less effectiveness of trastuzumab.

The direct link between gut microbiota and trastuzumab efficacy was demonstrated through an experiment of faecal microbiota reconstitution. The transplantation of faeces from control donors, but not from vancomycin donors, into ABX-treated FVB mice restored the efficacy of trastuzumab demonstrating the causality between commensal bacteria and the mAb activity in our animal model. The compromised recruitment of CD4⁺ T lymphocytes and GZMB⁺ cells in tumour of mice whose microbiota was reconstituted with faecal suspension from antibiotic-treated mice confirms the involvement of gut microbial ecosystem in trastuzumab-mediated triggering of host immune cells. Mice transplanted with faecal material from streptomycin-treated donors reproduced only partially the donor phenotype: besides an initial inhibition of trastuzumab activity they ended responding to the treatment. This is at least in part explained by the similarities between the gut microbiota of FMT-streptomycin mice and control mice observed at the end of the experiment likely due to the less severe alteration induced by streptomycin in the intestinal ecology as compared to those of vancomycin.

In this project we explored the possibility to use probiotic lactate-producing bacteria (LAB) to improve trastuzumab activity. In fact, in addition to a reduction in SCFAs levels, a common reduction in the amount of lactate in comparison to control mice was found and thus a beneficial potential of LAB was hypothesized. The concomitant administration of

Lactococcus lactis or *Lactobacillus paracasei* and trastuzumab improved its efficacy in mice under vancomycin regimens. To date, many studies reported the immune-enhancing activity of LAB species and strain like *Lactobacillus casei* Shirota has been reported to promote NK cells activation and Th1 immunity {Yasui, 2004}. However, no modification in intratumour immune cells frequencies was observed in mice gavaged with *L. lactis* or *L. paracasei*. This is probably due to the fact that the activation and spatial distribution of cells within the tumour microenvironment were not analysed and they probably would better reflect the impact of intestinal microbiota modification on tumour immune infiltrate than the simple analysis of immune subpopulation frequencies.

To translate the results obtained in animal studies to a clinical context, the content of the gut microbiota was analysed in HER2+ BCs treated with trastuzumab-based therapy and it revealed differences in association with the clinical response. Although the small number of patients in our cohort, the β -diversity analysis by PCoA was able to show a certain grade of separation between patients who achieved pCR and those who had residual disease at the time of the surgery. The achievement of pCR in HER2+ BC is associated with long-term outcome of patients and has been proposed as a surrogate endpoint for prediction of long-term clinical benefit such as DFS, EFS and OS {Cortazar, 2014}. Several studies have demonstrated strong association of specific gut microbiome signature with the response to immune checkpoint blockade, showing its potential value as predictor of patients clinical outcome with the ability to overcome other known biomarkers {Matson, 2018}. Together with microbiota composition, Dr Wargo's group {Gopalakrishnan, 2018} found that metastatic melanoma patients responsive to the anti-PD1 treatment had significantly higher alpha diversity compared to NR. In addition, R melanoma metastatic patients also clustered separately in the analysis of β -diversity by PCoA {Matson, 2018; Gopalakrishnan, 2018}. In our study, patients who achieved pCR presented different microbiota from non-responders: a prevalence of bacteria belonging to the *Clostridiales*

order characterized the gut microbiota of R patients, while higher abundance of *Bacteroidales* was found in NR as compared to R patients. Members of the *Lachnospiraceae*, *Turicibacteriaceae*, *Coriobacteriaceae* and *Prevotellaceae* taxonomic families were found less abundant in NR patients, as observed in antibiotic-treated mice, implying the relevance of these bacteria for trastuzumab benefit. Bacteria belonging to these taxonomic families are usually associated with homeostasis of the gut and a diet enriched in fibre {Zhao, 2018}.

The unsupervised clustering analysis identified two microbiota clusters that significantly discriminated patients according to response supporting predictive properties for gut communities in the treatment of HER2-positive BCs patients. Notably, when the PAM50 intrinsic molecular classification {Prat, 2014b} was applied to tumours, HER2-enriched cases were distributed in the two microbiota clusters based on trastuzumab response, supporting the contribution of gut microbiota in trastuzumab activity independently of tumour intrinsic molecular characteristics. Furthermore, the capability to recapitulate the response observed in clinical setting in mice transplanted with faecal material from R and NR patients, and the association of the unsupervised clustering analysis with the response to the treatment suggest the potential role of the gut microbiota as predictive biomarker. These findings advise that the gut microbiota should be considered in larger studies to assess its potential as independent predictor able to add information in the identification of those patients who benefit the most from the trastuzumab-based treatment.

Chapter 6: CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, our results demonstrate that trastuzumab antitumor activity is influenced by the gut microbiota composition, at least in tumours where the block of HER2 signalling is not sufficient to completely inhibit tumour growth . In fact, an intact intestinal microbiota is required for the fully efficacy of this mAb likely due to the ability of gut bacteria to shape host immunity.

In the FVB models, the alteration of intestinal microbiota by the use of a single antibiotic (vancomycin or streptomycin) abrogated the benefit of anti-HER2 treatment with a causal role for the microbiota. The same results were observed in FVB Δ 16HER2 transgenic mice, excluding the possibility that the presence of a strong immunogen, such as the human HER2, limited the interpretation of data in the syngeneic FVB transplantable model. Further, LAB administration was able to improve the sensitivity to trastuzumab in FVB under vancomycin treatment. In the BALB/c model, the single-antibiotic treatment and consequent intestinal microbiota modification did not impact the effectiveness of the 7.16.4 mAb. In a similar way, the depletion of the intestinal microbiota, although affecting the tumour immune infiltrate did not result in a diminished efficiency of the mAb underlying the importance of a high sensitivity of tumour cells to the treatment independently of the tumour microenvironment. Ongoing experiment in preclinical model will help to define the mechanisms by which the gut microbiota interferes with trastuzumab benefit, our studies will be then focused on the modification occurred in the tumour immune microenvironment upon alteration of the intestinal ecosystem. This represents a cogent challenge to design novel therapeutic approaches, other than the addition of other anti-HER2 agents or immunotherapy, for patients with HER2-positive BC who are resistant to trastuzumab.

In patients, despite the small number of cases, a differential microbiota abundance was identified between R and NR opening the possibility of using gut microbiota as predictive biomarkers. To validate our results in a larger cohort, we propose to enrolled

HER2-positive BC patients in both neoadjuvant and metastatic setting. Although determine the optimal sample size for our study is difficult due to the challenging statistical power and the unknown effect size analysis of the gut microbiota, we believe, by assuming a medium effect size of 0.5 for the gut microbiota on trastuzumab activity, that a minimum number of 40 patients should be enrolled in this study to compare the proportion of responders and non-responders according to gut microbiota composition using two sided Chi-square test with a type I error of 0.05. In addition, having a larger cohort of patients, will enable us to assess the association of the gut microbiota with other clinicopathological variables along with its predictive potential with respect to pCR evaluated through multivariate regression model that takes into consideration other already known biomarkers for trastuzumab response such as age, tumour size, nodal status, ER status and PAM50 classification. Particular attention will be paid for the antibiotic usage (high variability in the individual time needed for the recovery from changes occurred in the intestinal microbiota), storage condition (long period at room temperature may favour the increase of some bacteria more than other) and diet. These are three main sources of variability that we will be considered as confounders at the time of definition of control and exclusion criteria. For these reasons the use of antibiotics, the time passed from sampling to storage at -80°C and alimentary habits will be recorded and considered in future analyses.

The results obtained so far also represent the starting point to elaborate strategies aimed at the improvement of the therapeutic success in HER2+ BC. Interestingly, patients achieving pCR were characterized by high *Clostridiales* and low *Bacteroidales* relative abundances. Dietary interventions such as increasing the amount of fibre in the diet {Makki, 2018}, walnuts {Byerley, 2017}, or supplementation of prebiotic fibre {Umu, 2017} is able to promote the growth of these bacteria in the host and it would be very interesting in the future to investigate the beneficial effects of dietary intervention (or

pre/probiotics administration) on HER2-positive BC patients outcome. In support to the possibility to exploit the microbiota-immune axis in trastuzumab treatment, in the past, oral administration of TLR2 agonist was used to improve the immune-mediated antitumor efficacy of trastuzumab {Lu, 2011}. Similar to the ongoing study of the Wargo's group the potential benefit of a high fibre diet or the use of pre/probiotics can be investigated as an alternative strategy to boost the immune-mediated trastuzumab activity by fitting the gut microbiota composition.

Chapter 7: REFERENCES

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LIST OF COLLABORATIONS

This thesis includes results carried out with the support of valuable collaborations:

- Metagenomics analyses were conducted by Prof. Simone Giglielmetti and Dr Giorgio Garagari at the Department of Food, Environmental and Nutritional Sciences (DeFENS) of the Università degli Studi of Milan.
- Dr Stefania Arioli at DeFENS, Università degli Studi of Milan, performed flow cytometry analyses on faecal samples and provided *Lactococcus lactis* and *Lactobacillus paracasei* suspensions for *in vivo* treatment.
- The characterization of tumour immune infiltrate by IHC was carried out by the research group of Dr Claudio Tripodo at the Dept. of Human Pathology of the Università degli Studi of Palermo,
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- GEP of tumour biopsies and PAM50 classification were performed by Dr Loris De Cecco at the Platform of Integrated Biology, Department of Applied Research and Technology Development, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy.

PUBLICATIONS

Publications related to my PhD project

- **Di Modica M**, Gargari G, Regondi V, Bonizzi B, Arioli S, Belmonte B, De Cecco L, Tripodo C, Villani L, Corsi F, Guglielmetti S, Balsari A, Triulzi T and Elda Tagliabue E. Gut microbiota conditions the therapeutic efficacy of trastuzumab in HER2-positive breast cancer. **Manuscript submitted.**
- **Di Modica M**, Tagliabue E, Triulzi T. Predicting the Efficacy of HER2-Targeted Therapies: A Look at the Host. *Dis Markers*. 2017; 2017:7849108. doi: 10.1155/2017/7849108. Epub 2017 Dec 18. **Review.**

Published meeting abstracts

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